QUANTITATIVE ANALYSIS OF AMBROXOL HYDROCHLORIDE AND LORATADINE IN BULK AND PHARMACEUTICAL FORMULATIONS

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032.

In partial fulfillment of the requirements for the award of Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted

BY

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Reg.No.261330952

Under the guidance of

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DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

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CERTIFICATE

This is to certify that the dissertation entitled **QUANTITATIVE ANALYSIS OF AMBROXOL HYDROCHLORIDE AND LORATADINE IN BULK AND PHARMACEUTICAL FORMULATIONS"** submitted by **P.ARUMUGAM** (Reg No: 261330952) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in Department of Pharmaceutical Analysis, Edayathangudy G.S.Pillay College of Pharmacy, Nagapattinam during the academic year 2014-2015.

Prof.Dr.D.BabuAnanth,M.Pharm., Ph.D.,

Place: Nagapattinam

Date:

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1. INTRODUCTION

Everything made by human hands is subject to decay. Pharmaceuticals are no exception to this. A drug for oral use may destabilize either during its shelf life or in the GIT. Two major stability problems resulting in poor bioavailability of an orally administered drugs are – degradation of drug in to inactive form, and because of interaction with one or more different components present in dosage, which form a complex in GIT that may be poorly soluble or unabsorbable.[1]

Stability testing forms an important part of the process of drug product development. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, and suggest recommendation of storage conditions, retest periods, and shelf lives, that need to be established. The two main aspects of drug product, that play an important role in shelf life determination are assay of active drug, and degradants generated, during the stability study. The assay of drug product in stability test sample needs to be determined using stability indicating method, as recommended by the International Conference on Harmonization (ICH) guidelines[2] and USP 26.[3]

Fixed dose combination containing Ambroxol Hydrochloride (60mg) and Loratadine (5mg) is available in tablet form in the market. This combination therapy was shown to be superior used to treat respiratory disorder and allergies condition. Analytical research and development of fixed dose combination is found to be very interesting and challenging job, hence development of stability indicating method for Ambroxol Hydrochloride and Loratadine in combination has been selected for the present study.

2. OBJECTIVES

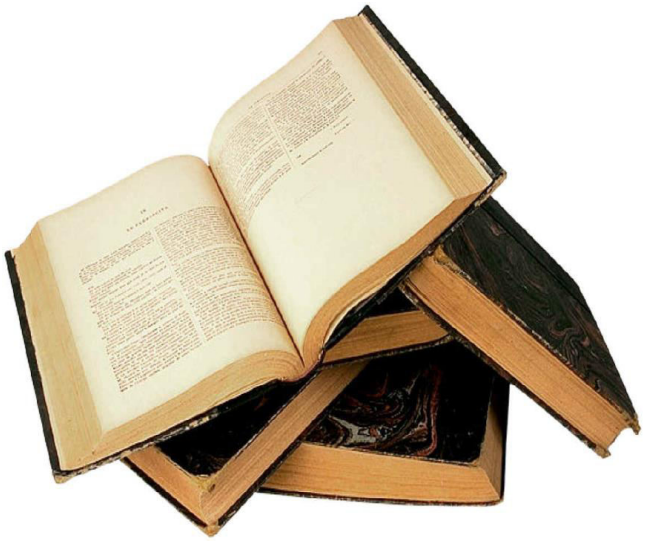
2. OBJECTIVE

The objective of this work was the stability indicating HPLC method for simultaneous estimation of Ambroxol HCL and Loratadine in pharmaceutical formulation. Particular goals were:

- To develop a HPLC method for simultaneous estimation of Ambroxol HCl and Loratadine.
- To validate the method developed using parameters like accuracy, precision, linearity and range for the estimation of these drugs in pharmaceutical dosage form.
- To obtain the stress degraded products of Ambroxol HCl and Loratadine by exposing a formulation which is under study for different stress conditions like acid, base, oxidative, reductive and neutral media.
- To study the stress degradation behavior of Ambroxol HCl and Loratadine by analyzing the different products obtained after degradation using HPLC method.
- To apply the stability indicating HPLC method for the simultaneous estimation of Ambroxol HCL and Loratadine, and degraded product in a pharmaceutical formulation.

3.

REVIEW OF LITERATURE



3. REVIEW OF LITERATURE

3.1. DRUG PROFILE

- AMBROXOL HYDROCHLORIDE
- LORATADINE

3.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

3.3. ANALYTICAL METHOD VALIDATION

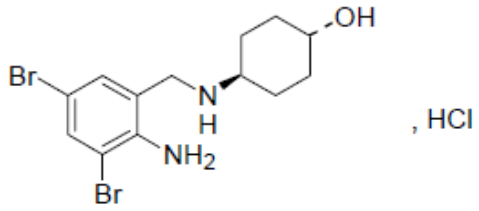
3.4 STABILITY INDICATING METHOD

3.5. LITERATURE SURVEY

3.1. DRUG PROFILE:

- **AMBROXOL HYDROCHLORIDE**

Table3.1 Drug Profile of Ambroxol Hydrochloride[4, 5]

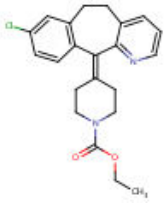
CAS Registry number	18683-91-5
Generic Name	**Ambroxol hydrochloride**
Category	Mucolytic agent
Dosage forms	Tablets (30 mg, 60 mg) Oral liquid formulation (15 mg/5 ml)
Brand names	1.Acocontin 2.Acolyt 3.Ambrodil 4.Cetry puls
Chemical structure	 , HCl
Chemical name	*trans*-4-[(2-amino-3,5 dibromobenzyl)amino] cyclohexanol hydrochloride
Molecular formula	$C_{13}H_{18}Br_2N_2O$,HCl
Molecular Wt (g/mol)	414.6
Ionization constant	7.1,8.2
Appearance	A white or yellowish crystalline powder.
Melting Point range	235 - 240 ˚C
Loss on Drying	Not more than 0.5%
Heavy Metals (ppm)	Not more than 20 ppm
Total impurity	Not more than 0.50%
Residue on ignition	Not more than 0.10%

Solubility	Sparingly soluble in *water*; soluble in *methanol*; practically insoluble in *methylene chloride.*
BCS Class	I (one)
Indications and Uses	• Acute and Chronic disorders of the respiratory tract associated with pathologically thickened mucus and impaired mucus transport. • Its relieving pain in acute sore throat.

- **LORATADINE**

Table 3.2 Drug Profile of Loratadine [6, 7]

CAS Registry number	79794-75-5
Generic Name	**Loratadine**
Synonyms	• Loratadina [Spanish] • Loratadinum [Latin]
Category	• Antipruritics • Anti-Allergic Agents • Antihistamines • Histamine H1 Antagonists, Non-Sedating
Dosage forms	10 mg, 30 mg, 40 mg tablet dosage forms
Brand names	• Claritin • Claritin Reditabs • Claritin-D • Claritine • Clarityn • Clarityne

Chemical structure	
Chemical name	ethyl 4-{13-chloro-4-azatricyclo[9.4.0.0^{3,8}]pentadeca-1(11),3,5,7,12,14-hexaen-2-ylidene}piperidine-1-carboxylate
Molecular formula	$C_{22}H_{23}ClN_2O_2$
Molecular Wt (g/mol)	382.883
Ionization constant	4.9
Appearance	A white solid powder
Melting Point range	134-136 ˚C
Loss on Drying	Not more than 0.2
Heavy Metals (ppm)	Not more than 10
Total impurity	Not more than 0.30%
Residue on ignition	Not more than 0.1%
Solubility	very soluble in acetone, alcohol, and chloroform.
BCS Class	II (two)
Therapeutic category	Antihistamic Agent (H1 blocker)
Indications and Uses	• A self-medication that is used alone or in combination with pseudoephedrine sulfate for the symptomatic relief of seasonal allergic rhinitis. • Also used for the symptomatic relief of pruritus, erythema, and urticaria associated with chronic idiopathic urticaria in patients.

3.2. HIGH PERFOMANCE LIQUID CHROMATOGRAPHY

Although there is spectacular advancement in the instrumental methods of analysis, the success or failure of such method largely depends upon how pure is the sample for such analysis. This is because a light impurity in analyte will lead to erroneous results. So large number of separation methods were discovered to isolate analytical species before any instrumental method is resorted. Such separation methods included not only chromatographic methods but the non-chromatographic techniques like solvent extraction, ring oven; zone refining, froath floation, dialysis, reversed osmosis and precipitation methods. However, chromatographic methods have become most popular because of the simplicity and cost of analysis. The entire credit for popularizing chromatography technique for the separation goes to HPLC and advanced techniques of HPLC.

HPLC is a physical separation technique carried out in the liquid phase in which a sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). An online detector monitors the concentration of each separated component in the column effluent and generates a chromatogram. HPLC is the most widely used analytical technique for the quantitative analysis of pharmaceuticals, biomolecules, polymers, and other organic compounds.[9]

❖ **Principle of High Performance Liquid Chromatography[8, 9]**

Normal-Phase Chromatography

Normal-phase HPLC explores the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase. The stronger the analyte stationary phase interaction, the longer the analyte retention. As with

any liquid chromatography technique, NP-HPLC separation is a competitive process. Analyte molecules compete with the mobile-phase molecules for the adsorption sites on the surface of the stationary phase. The stronger the mobile-phase interactions with the stationary phase, the lower the difference between the stationary-phase interactions and the analyte interactions, and thus the lower the analyte retention. Mobile phases in NP-HPLC are based on nonpolar solvents (such as hexane, heptane, etc.) with the small addition of polar modifier (like methanol, ethanol). Variation of the polar modifier concentration in the mobile phase allows for the control of the analyte retention in the column. Typical polar additives are alcohols (methanol, ethanol, or isopropanol) added to the mobile phase in relatively small amounts. Since polar forces are the dominant type of interactions employed and these forces are relatively strong, even only 1 % v/v variation of the polar modifier in the mobile phase usually results in a significant shift in the analyte retention.

Reversed Phase Chromatography

As opposed to normal-phase HPLC, reversed-phase chromatography employs mainly dispersive forces (hydrophobic or Van der Waals interactions). The polarities of mobile and stationary phases are reversed, such that the surface of the stationary phase in RP-HPLC is hydrophobic and mobile phase is polar, where mainly water- based solutions are employed. RP-HPLC is by far the most popular mode of chromatography. Almost 90% of all analyses of low-molecular-weight samples are carried out using RP HPLC. One of the main drivers for its enormous popularity is the ability to discriminate very closely related compounds and the ease of variation of retention and selectivity. The origin of these advantages could

be explained from an energetic point of view: Dispersive forces employed in this separation mode are the weakest intermolecular forces, thereby making the overall background interaction energy in the chromatographic system very low compared to other separation techniques. This low background energy allows for distinguishing very small differences in molecular interactions of closely related analyte.

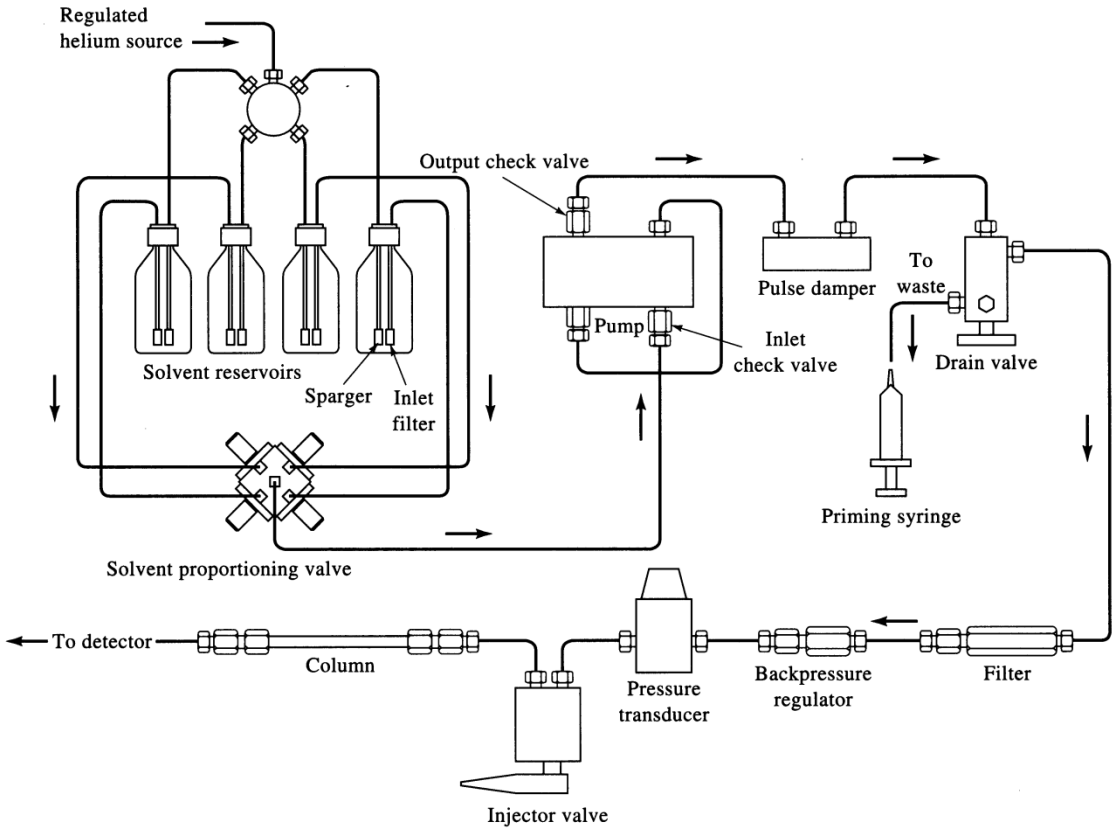


Fig: 3.1 Schematic Diagram of HPLC Instrument

HPLC, a sophistication chromatography technique is most widely used of all analytical separation techniques. Typical HPLC system the liquid mobile phase is forced through the stationary phase under pressure. It includes a solvent reservoir to hold the mobile phase, a pump to pressurize the mobile phase, and injector to allow injection of a small volume of the sample mixture under high pressure, a

column containing the bed of stationary phase, a detector to detect the presence of components as they exit the column, and a recorder to record the detector signal.

❖ **Basic Chromatographic Descriptors[9, 10]**

Following major descriptors are commonly used to report characteristics of the chromatographic column, system, and particular separation:

1. Capacity factor or Retention factor (k)
2. Efficiency (Plate number, N)
3. Resolution (R)
4. Separation factor (Selectivity, α)
5. Tailing factor (T) or asymmetry factor (As)

1. Capacity factor or Retention factor

Retention factor (k) is the unit less measure of the retention of a particular compound in a particular chromatographic system at given conditions defined as

$$k = \frac{V_R - V_O}{V_O} = \frac{t_R - t_O}{t_O}$$

Where V_R is the analyte retention volume, V_0 the volumes of the liquid phase in the chromatographic system, t_R the analyte retention time, and t_0 sometimes defined as the retention time of non-retained analyte.

Retention factor is convenient, since it is independent of the column dimensions and mobile phase flow rate. Note that all other chromatographic conditions significantly affect analyte retention.

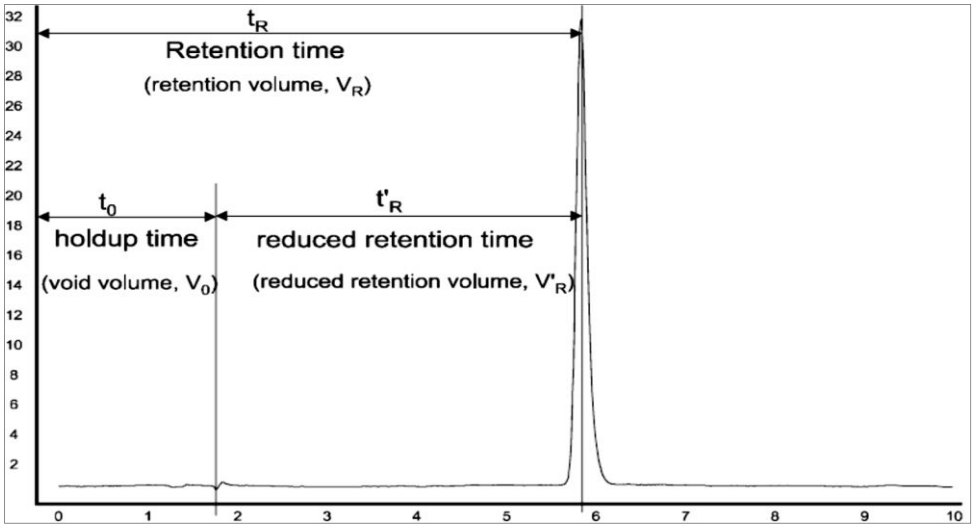


Fig: 3.2 Analyte Retention Descriptors

2. Efficiency (Plate number, N)

Efficiency is the measure of the degree of peak dispersion in a particular column, as such it is essentially the characteristic of the column. Efficiency is expressed as the number of theoretical plates (N) calculated as

$$N = 16\left(\frac{t_R}{w}\right)^2$$

Where t_R is the analyte retention time and w the peak width at the baseline.

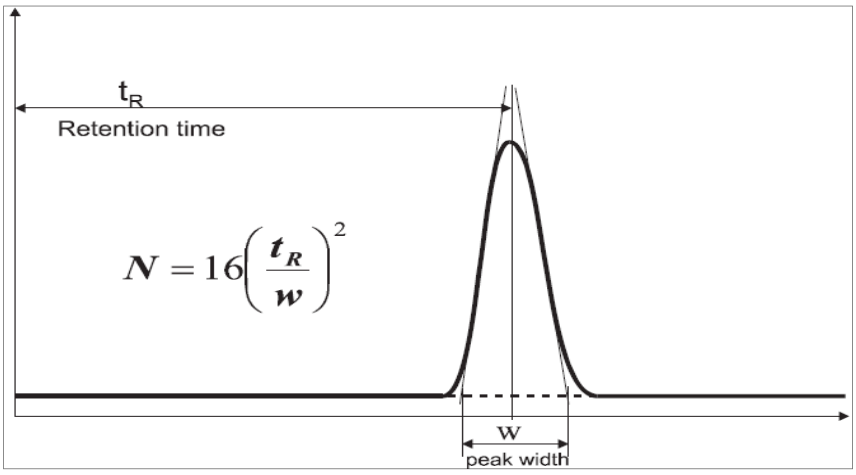


Fig: 3.3 Schematic Diagram of Efficiency Measurements (Number of Theoretical Plates in the Column)

3. Resolution (R)

It is a measure of quality of separation of adjacent bands in a chromatogram; obviously overlapping bands have small R values. It is calculated from the width and retention time of two adjacent peaks.

$R = 2(t_2 - t_1)/w_1+w_2$

Where, t_1 and t_2 are the retention time of first and second adjacent bands; w_1 and w_2 are widths at their baseline.

Reliability of calculation is poor if R is < 1.0.

4. Separation Factor (Selectivity) (α)

Selectivity (α) is the ability of chromatographic system to discriminate two different analytes. It is defined as the ratio of corresponding capacity factors

$\alpha = k_2/k_1 = t_{R2} - t_0 / t_{R1} - t_0$

5. Tailing Factor (T) or Asymmetric Factor (As)

The tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. In some cases values less than unity may be observed. As peak asymmetry increases and hence precision becomes less reliable.

It is expressed as-

$T = w_{0.05} /2d$

$w_{0.05}$ is width of peak at 5 % height and d = half of peak width at 5% peak height. Ideally the T value should be ≤ 2.

❖ **Strategy for Method Development in HPLC[8-13]**

Everyday many chromatographers face the need to develop a high-performance liquid chromatography (HPLC) separation. Method development and optimization

in liquid chromatography is still an attractive field of research for theoreticians (researchers) and attracts also a lot of interest for practical analysts. Complex mixtures or samples required systematic method development involving accurate modeling of the retention behavior of the analyte. Among all, the liquid chromatographic methods, the reversed phase systems based on modified silica offers the highest probability of successful results. However, a large number of (system) variables (parameters) affect the selectivity and the resolution.[10]

HPLC method development follows a series of steps, which are summarized as below:

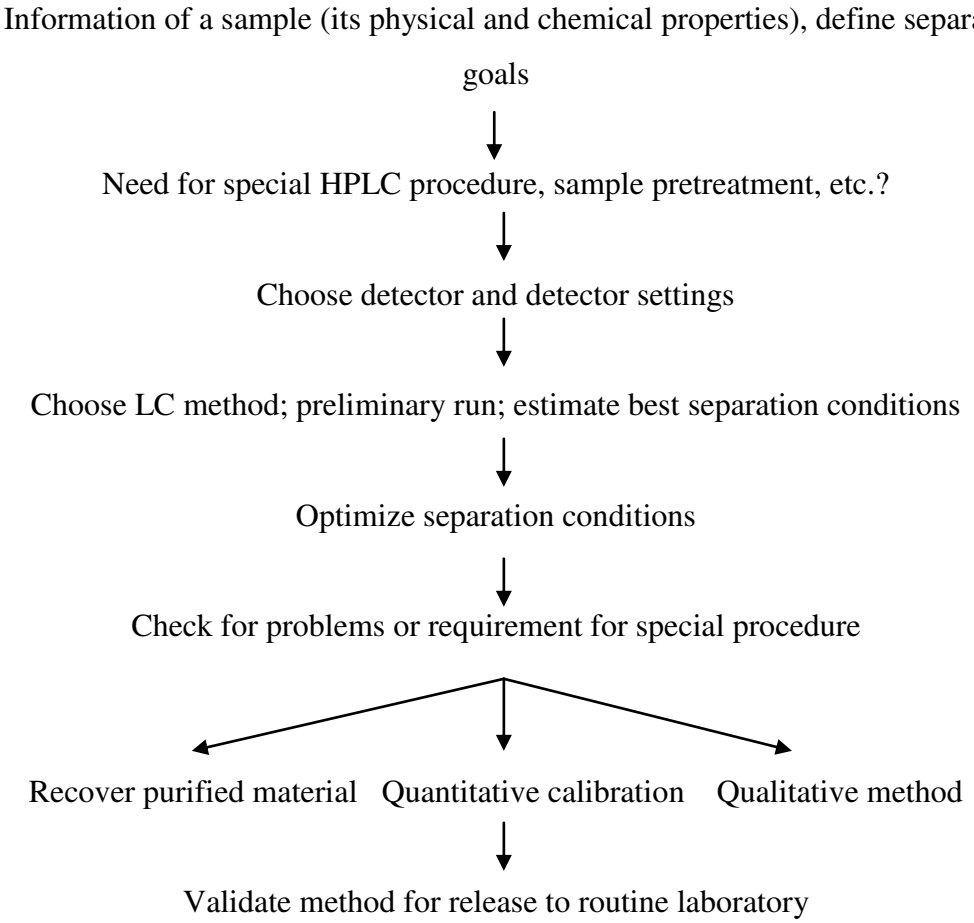


Fig: 3.4 Steps in HPLC Method Development[10]

HPLC method development is not very difficult when a literature reference for the same or similar compounds to be analyzed can found.

1) Nature of Sample[10]

Before proceeding with development of method for a particular sample, it is absolutely essential to have detailed information about sample. What are the components present? Excipients and impurity present in sample must be identified.

Some important information concerning sample are:

1. Number of components present.
2. Chemical structures (functionality), molecular weight, pKa and solubility of compounds.
3. UV spectra of compounds.
4. Concentration range of compounds in samples of interest.

2) Separation Goal[10]

The goals of HPLC separation need to be specified clearly. Some related questions that should be asked at the beginning of method development include:

1. Is the primary goal quantitative analysis, the detection of a substance, the characterization of unknown sample components or the isolation of purified material?
2. Is it necessary to resolve all sample components?
3. If quantitative analysis is required, what levels of accuracy and precision are required?
4. For how many different sample matrices should the method be designed?
5. How many samples will be analyzed at one time?

3) Sample Pre-treatment[10]

Sample pre-treatment is very important in development of a new method. Most of sample required dilution before injection. Samples come in various forms:

1. Solution ready for injection.
2. Solution requires dilutions, buffering and addition of an internal standard.
3. Solid that must be dissolved or extracted.
4. Samples that require sample pretreatment to remove interference and /or to protect the column or equipment from damage.

   Direct injection of the sample is preferred for its convenience and greater precession. Best result are obtained when concentration of sample solvent are same as mobile phase. Nature and concentration as samples are very important because concentrated analyte can damage the column.

4) Detector and Detector Settings[10]

Variable-wavelength ultraviolet (UV) detectors normally are the first choice, because of their convenience and applicability for most samples. For this reason, information on UV spectra can be an important aid for method development. UV spectra can found in the literature, estimated from chemical structures of sample components of interest, measured directly (if pure compounds are available), or obtained during HPLC separation by means of photodiode-array detector. To obtain better sensitivity detection should be carried out at the absorption maximum of the substance. Universal detection is possible at 210 nm where purity of acetonitrile is important.

5) Developing the Separation[14]

The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of analyte will allow the most appropriate mode of HPLC to be selected. For the selection of a suitable

chromatography method for organic compounds first Reversed-phase should be tried, if not successful, normal-phase should be taken into consideration.

Phase selection process should be followed as shown in figure 3.5 considering the sample characteristics.

❖ **Reversed Phase Chromatography[9]**

Eluent Choice

In Reversed Phase Chromatography, acetonitrile is the preferred organic solvent because of low viscosity and high UV transparency (if pure); disadvantage being poisonous, expensive.

Aqueous eluent preferred are water: for neutral compound, 10 mM H_3PO_4, pH 2.3: for weak to medium acids (ion suppression), 10 mM phosphate buffer, pH 4.0: for weak to medium acids (partly ion suppression), 5 mM phosphate buffer, pH 7.5: for weak to medium bases or acids in ionization form, Unknown sample should be analyzed first with water, then with an acid and a neutral buffer: acid and basic compounds can be recognized by change of retention time.

Eluent's Choice

According to eluotropic sequence (the UV-transparency must be taken into consideration) in Normal Phase Chromatography. n-hexane/dioxane can be used nearly universally. Eg. amides, sulfonamides, nitro compounds, heterocycles,

carbamates, urea and alcohols can be eluted successfully in n-hexane/dioxane system.

This system equilibrates fast and is stable; the water content of the eluent has no influence on the retention anymore.

1. Weaker eluents are n-hexane/CH_2Cl_2 or pure n-hexane for hydrocarbons, compounds with non-polar groups as esters, ethers and stronger eluents as n-hexane/ isopropanol for polar compounds as carboxylic acids.

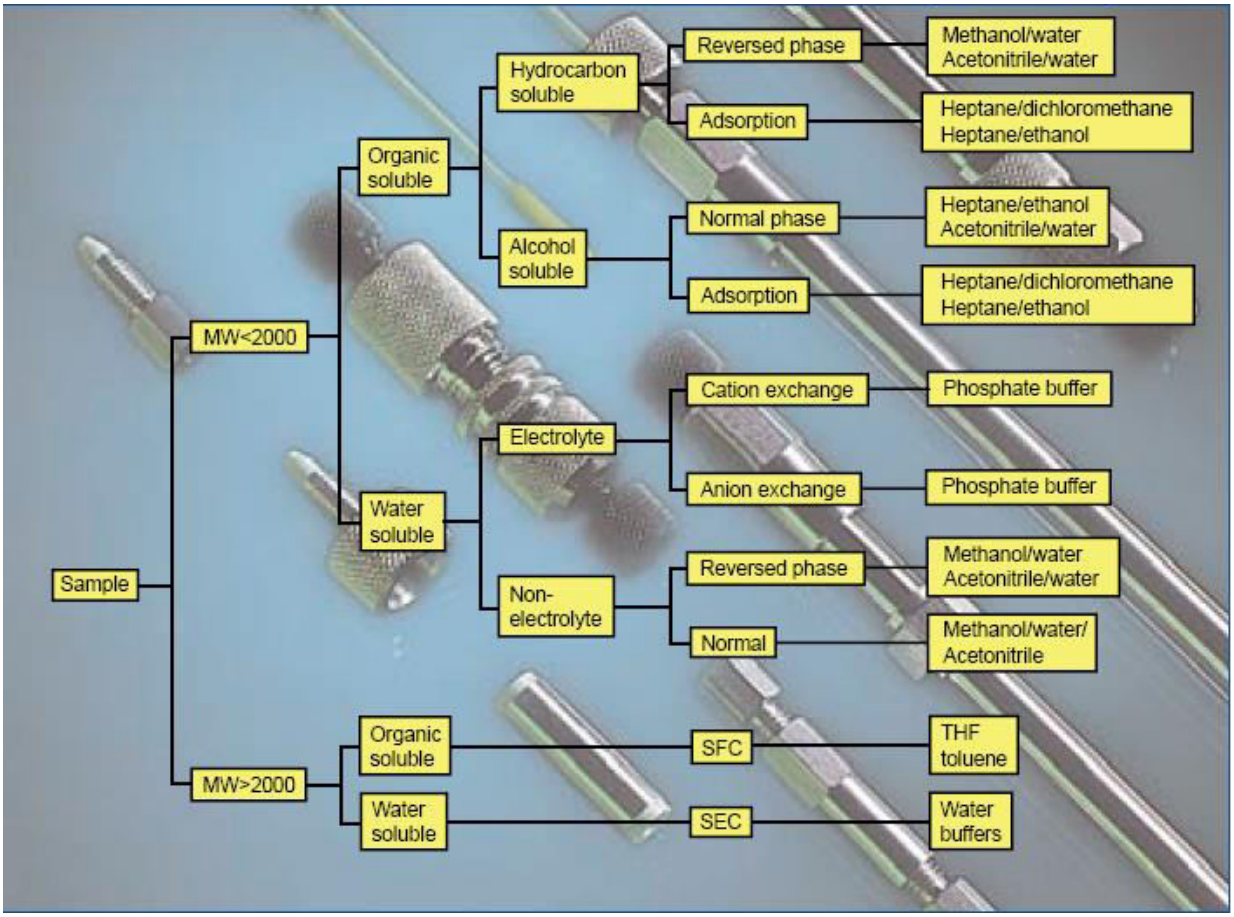


Fig: 3.5 Phase Selection Process

Specifically, the experienced chromatographer will consider several aspects of the separation, as summarized in Table 3.3

Table: 3.3 Preferred Experimental Conditions for the Initial HPLC Separation[9]

Separation Variable		Preferred initial choice
Column	Dimensions(Length × ID)	15 × 0.46 cm
	Particle Size	5µm
	Stationary Phase	C_8 or C_{18}
Mobile Phase	Solvents A and B	Buffer-Acetonitrile
	% B	80-100%
	Buffer (compound, pH, concentration)	10 - 25mM Phosphate Buffer 2.0 < pH< 3.0
	Additives(e.g., amine modifiers, ion-pair reagents)	Do not use initially
	Flow-rate	1.5-2.0 ml/min
	Temperature	35 – 45°C
Sample Size	Volume	< 25 µl
	Weight	< 100 µg

Peak shape is often a problem, especially for basic compounds analyzed by reversed phase HPLC. To minimize any potential problems always use a high purity silica phase such as Wakosil II. These modern phases are very highly deactivated so secondary interactions with the support are minimal. Buffers can be used effectively to give sharp peaks. If peak shape remains a problem, use an organic modifier such as triethylamine.[10]

When separating acids and bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. For basic or cationic samples, "less acidic" reverse-phase columns are recommended and amine additives for the mobile phase may be beneficial. Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Beyond that, buffering capacity will be inadequate. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols. To be most

effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.[15]

The pH range most often used for reversed-phase HPLC is 1 - 8 and can be divided into low pH (1 - 4) and intermediate pH (4 - 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is, maximized. For this reason, operating at low pH is recommended. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pKa values. A more rugged mobile phase pH will be at least 1 pH unit different from the analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography. Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed.

Table: 3.4 System Suitability Parameters and Recommendations

Parameter	Recommendation
Capacity Factor (k)	The peak should be well resolved from other peaks generally k >2.0
Repeatability	RSD ≤ 1% for n ≥ 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R)	R > 2 between the peak of interest and the closest Eluting potential interference (impurity, excipient, degradation product, internal standard) etc.
Tailing Factor (T)	T ≤ 2
Theoretical Plates (N)	In general should be > 2000

❖ **Quantitative Analysis by HPLC[15]**

Quantitative column chromatography is based upon a comparison of either the height or the area of the analyte peak with that of one or more standards.

1. Analyses Based on Peak Height

The height of a chromatographic peak is obtained by connecting the base lines on either side of the peak by a straight line and measuring the perpendicular distance from this line to peak. It is important to note, however, that peaks height are inversely related to peak widths. Thus, accurate results are obtained with peak heights only if variations in column conditions do not alter the peak widths during the period required to obtain chromatogram for sample and standards.

2. Analyses Based on Peak Areas

Most modern chromatographic instruments are equipped with digital electronic integrators that permit precise estimation of peak areas. A simple method, which works well for symmetric peaks of reasonable widths, is to multiply the height of peak by its widths at one half the peak heights. Other methods involve the use of planimeter or cutting out the peak and determining its weight relative to the weight of a known area of recorded paper.

3. Calibration and Standards

The most straight forward method for quantitative analysis involves the preparation of series of standard solutions that appropriate the composition of the unknown. Chromatograms for the standards are then obtained and peak heights or areas are plotted as function of concentration.

4. Area Normalization Method

After integrating all significant peaks in a chromatogram, total peak area may be calculated. Area (%) of any individual peak is called normalized peak area. This technique is widely used particularly in preliminary method development.

$$\% \text{ A} = \frac{\text{Area of Peak A}}{\text{Total Area of Peaks (A + B + C + D)}} \times 100$$

5. Internal Standard Method

The highest precision for quantitative chromatography is obtained by use of internal standard because the uncertainties introduced by sample injection are avoided. In this procedure, a carefully measured quantity of an internal standard substance is introduced in to each standard and sample, and the ratio of analyte to internal standard peak areas (or height) serves as the analytical parameters. Addition of IS is essential for the sample requiring significant pre-treatment such as derivatisation, extraction to reduce chances of error due to these steps as it is expected to mimic the behaviour of analyte in such re-treatment steps. A calibration curve is produced by analyzing different concentrations of the pure drug with constant amount of IS from the chromatogram and calculate the ratio (Rs) for each concentration of the analyte.

$$\text{Rs} = \frac{\text{Area of the Drug}}{\text{Area of the Internal Standard}}$$

Plot this ratio against concentration of the pure drug. The slope of this plot is the response factor.

The requirements for internal standards are that; it must completely resolve peak (R>1.25) with no interferences. It should elute close to the compound of interest and behave equivalent to the compound of interest for analysis like pretreatments

and derivative formations. It should also be stable, unreactive with sample components, column packing and the mobile phase and commercially available in high purity. This technique gives reliable, accurate, and precise results. If the internal standard is truly inert, the method is useful for determining the rate of analyte conversion in a chemical reaction.

3.3. ANALYTICAL METHOD VALIDATION [16-18]

Method validation, according to United States Pharmacopoeia, is performed to ensure that an analytical methodology is accurate, specific, reproducible, and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use and is sometime described as the process of providing documented evidence that the method does what it is intended to do. Regulated laboratories must perform method validation in order to be in compliance with FDA regulations.

I. Accuracy

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is measured as the percentage of analyte recovered by assay, by spiking samples in a blind study.

To document accuracy, the ICH guideline on methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range(for example, three concentrations with three replicates each). The data should be reported as the percentage recovery of the known, added amount, or as the difference between mean and true value with confidence intervals.

II. Precision

ICH defines the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra–assay precision.

Intermediate precision expresses variations within laboratories, such as different days, different analysts, different equipment, and so forth.

Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology).

The ICH requires repeatability to be tested from at least six replications measured at 100 percent of the test target concentration or from at least nine replications covering the complete specified range. For example, the results can be obtained at three concentrations with three injections at each concentration.

III. Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. It is a measure of degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due only to a single component, that is, that no co-elutions exist. Specificity is measured and documented in a separation by the resolution, plate count (efficiency), and tailing factor.

ICH divides the term specificity into two separate categories: identification and assay/impurity tests. For identification purposes, specificity is demonstrated by the ability to discriminate between compounds of closely related structures, or by comparison to known reference materials. For assay and impurity tests, specificity is demonstrated by the resolution of the two closest eluting compounds. The compounds are usually major component or active ingredient and an impurity. If the impurities are available, it must be demonstrated that the assay is unaffected by the presence of spiked materials (impurities and /or excipients). If impurities are not available, the test results are compared to a second well characterized procedure. For assay tests, the two results are compared; for impurity tests, the impurity profiles are compared head to head.

IV. Linearity and Range

ICH defines linearity of an analytical procedure as its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in a sample.

Linearity may be demonstrated directly on the test substance (by dilution of standard stock solution) or by separately weighing synthetic mixtures of the test product components.

Linearity is determined by series of five to six injections of five or more standards whose concentrations span 80-120 percent of the expected concentration range. The response should be directly proportional to the concentration of analyte or proportional to the well- defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different

from zero. If a significant non zero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

Frequently, the linearity is evaluated graphically, in addition to or as an alternative to mathematical evaluation. The evaluation is made by visually inspecting a plot of single height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used. The first is to plot deviations from regression line versus the concentration or versus the logarithm of the concentration if the concentration range covers the several decades. For linear ranges, the deviation should be equally distributed between positive and negative values.

Another approach is to divide single data by their respective concentrations, yielding the relative responses. A graph is plotted with the relative response on y-axis and the corresponding concentrations on the x-axis, on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn on the graph corresponding to, for examples, 95 percent and 105 percent of the horizontal line. The method is linear up to the point where the plotted relative response line intersect the 95 percent line.

V. Limit of Detection

The limit of detection is defined as the lowest concentration of the analyte in the sample that can be detected, though not necessarily quantitated. It is the limit test that specifies whether or not an analyte is above or below a certain value. LOD may be calculated based on the standard deviation (SD) of the response and the slope(S) of the calibration curve at levels approaching the LOD according to the

formula: LOD = 3.3(SD/S). The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y- intercepts of regression lines. The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

VI. Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The calculation is based on the standard deviation (SD) of the response and the slope (S) of the calibration curve according to the formula LOQ = 10(SD/S).Again, the standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or standard deviation of y-intercepts of regression lines. As with LOD, the method used to determine LOQ should be documented and supported, and an appropriate number of samples should be analyzed at the limit to validate the level.

VII. Ruggedness

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as % relative standard deviation (RSD). These conditions include differences in laboratories, analyst, instruments, reagents, and experimental periods.

VIII. Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as percentage organic solvent, pH, ionic strength, or temperature, and determining the effect (if any) on the results of the method.

IX. System suitability

System suitability tests are most often applied to analytical instrumentation. They are designed to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronic, analytical operation and sample constituent an integral system that can be evaluated as a whole.

3.4 STABILITY INDICATING ASSAY METHOD: [19-22]

The stability-indicating assay is a method that is employed for the analysis of stability of samples in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products.[20, 21]

Stability-indicating methods according to United States-Food and Drug Administration (US-FDA) stability guideline of 1987 were defined as the 'Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.' This definition in the draft guideline of 1998 reads as: 'Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference. [22]

Types of stability indicating assay method (SIAM) [20]

a) Specific Stability Indicating Assay Method

It can be defined as 'A method that is able to measure unequivocally the drug(s) in the presence of all degradation products, in the presence of excipients and additives, expected to be present in the formulation.'

b) Selective Stability Indicating Assay Method

Whereas it can be defined as 'A method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation'.

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ASSAY METHODS (SIAMs):[20, 23-31]

Step I: Study of the drug structure

Major information about the drug can be gained from the structure, by studying of the functional groups, their way of degradation and other key components. There are defined functional group categories, like amides, esters, lactams, lactones, etc. that undergo hydrolysis, others like thiols, thioethers, etc. undergo oxidation, and compounds like olefins, aryl halo derivatives, aryl acetic acids, and those with aromatic nitro groups, N-oxides undergo photo decomposition.[27]

Step II: Data of physicochemical parameters of drugs

To start with the method development, it is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question. The knowledge of pKa is important as most of the pH- related changes in retention time depend on the pH of the buffer to be used in the mobile phase. The knowledge of log P of the drug and the identified degradation products provides good insight into the separation behaviour likely to be obtained on a particular stationary phase.

Step III: Stress (forced decomposition) studies.[28]

Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of

the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. The standard conditions for photostability testing are described in ICH Q1B.

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures. However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions. Results from these studies will form an integral part of the information provided to regulatory authorities.

Step IV: Preliminary separation studies of stressed samples[30]

The stress samples so obtained are subjected to preliminary analysis to study the number and types of degradation products formed under various conditions. For doing so, the simplest way is to start with a reversed-phase octadecyl column, preferably a new or the one in a healthy condition. Well-separated and good quality peaks at the outset provide better confidence because of the unknown nature of products formed during stressing. It should

be preferred to use water-methanol or water-acetonitrile as the mobile phase in an initial stage.

Step V: Final method development and optimizatin[31]

To separate close or co-eluting peaks, the method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type.

Step VI: Identification and characterization of degradation products

From this data, one can do the structure elucidation of the degraded product study. This can be done by using model analytical technique like LC-MS, GS-MS, H^1NMR, C^{13}NMR, IR.

Step VI: Validation of Stability Indicating Assay Methods

The main focus of validation is on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, etc. The limits of detection and quantitation are also determined which is having application in the analysis of stability of samples of bulk drug for determination of its expiry period. In the second stage, when the developed SIAM is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents.

3.5. LITERATURE SURVEY

- Krishna Veni Nagappan et al developed RP-HPLC method for simultaneous estimation of Ambroxol Hydrochloride and Loratidine in pharmaceutical formulation. The method was carried out on a Phenomenex Gemini C18 (25 cm x 4.6 mm i.d., 5 µm) column with a mobile phase consisting of acetonitrile: 50mM Ammonium Acetate (50:50 v/v) at a flow rate of 1.0 ml/min. Detection was carried out at 255 nm. Hydrochlorthiazide was used as an internal standard. The retention time of Ambroxol Hydrochloride, Loratidine and Hydrochlorthiazide was 5.419, 15.549 and 3.202 min, respectively. The linear ranges were from 3.0-21.0 to 0.250-1.750 µg/ml for Ambroxol Hydrochloride and Loratadine, respectively. The percentage recovery obtained for Ambroxol Hydrochloride and Loratadine were 99.78 and 99.20%, respectively.[32]
- K.A. Shaikha et al developed and validated a reversed-phase HPLC method for simultaneous estimation of Ambroxol hydrochloride and Azithromycin in tablet dosage form. The chromatographic separation was achieved on a Xterra RP18 (250mm×4.6mm, 5µm) analytical column. A Mixture of acetonitrile–Dipotassium phosphate (30mM) (50:50, v/v) (pH 9.0) was used as the mobile phase, at a flow rate of 1.7 ml/min and detector wavelength at 215 nm. The retention time of Ambroxol and Azithromycin was found to be 5.0 and 11.5 min, respectively.[33]
- Krupa M. Kothekar et al developed and validated analytical method for quantitative determination of Levofloxacin and Ambroxol hydrochloride in a new tablet formulation. Chromatographic separation of the two drugs was achieved on a Hypersil BDS C18 column (25cm X 4.6mm, 5µm). The mobile

phase constituted of Buffer: Acetonitirile: Methanol (650:250:100, v/v/v) with triethylamine and pH adjusted to 5.2 with dilute orthophosphoric acid was delivered at the flow rate of 1.0 ml/min. Detection was performed at 220 nm. Separation was completed within 10min. The linear dynamic ranges were from 30–180 to 250–1500 µg/ml for Ambroxol Hydrochloride and Azithromycin, respectively. The percentage recovery obtained for Ambroxol Hydrochloride and Azithromycin were 99.40 and 99.90%, respectively. Limit of detection and quantification for Azithromycin were 0.8 and 2.3µg/ml, for Ambroxol Hydrochloride 0.004 and 0.01 µg/ml, respectively.[34]

- Silvia Imre et al developed a new sensitive and selective liquid chromatography coupled with mass spectrometry (LC/MS/MS) method for quantification of Loratadine (LOR) and its active metabolite Descarboethoxyloratadine (DSL) in human plasma, After addition of the internal standard, metoclopramide. The human plasma samples (0.3 ml) were precipitated using acetonitrile (0.75 ml) and the centrifuged supernatants were partially evaporated under nitrogen at 37ºC at approximately 0.3 ml volume. The LOR, DSL and internal standard were separated on a reversed phase column (Zorbax SB-C18, 100mm×3.0mm i.d., 3.5µm) under isocratic conditions using a mobile phase of an 8:92 (v/v) mixture of acetonitrile and 0.4% (v/v) formic acid in water. The flow rate was maintained at 1 ml/min and the column temperature was kept at 45ºC. The detection of LOR, DSL and internal standard was done in MRM mode using an ion trap mass spectrometer with electrospray positive ionization. The ion transitions were monitored as follows: 383→337 for LOR, 311→(259 + 294 + 282) for DSL and 300→226.8 for internal standard.[35]

- Meiling Qi et al determined of Roxithromycin and Ambroxol Hydrochloride in a new tablet formulation by liquid Chromatography. Chromatographic separation of the two drugs was achieved on a Diamonsil™ C18 column (200mm×4.6 mm, 5µm). The mobile phase consisting of a mixture of acetonitrile, methanol and 0.5% ammonium acetate (39:11:50 v/v/v, pH 5.5) was delivered at a flow rate of 1.0 ml/min. Detection was performed at 220 nm. Linearity, accuracy and precision were found to be acceptable over the concentration range of 201.2–2012.0µg/ml for Roxithromycin and 42.7–427.0µg/ml for Ambroxol Hydrochloride, respectively.[36]
- Nilgun Gunden Goger et al worked on quantitative determination of Ambroxol in tablets by derivative UV spectrophotometric method and HPLC. Determination of Ambroxol in tablets was conducted by using first-order derivative UV spectrophotometric method at 255 nm (n=5). Standards for the calibration graph ranging from 5.0 to 35.0 µg/ml were prepared from stock solution. The proposed method was accurate with 98.69 -100.69% range of recovery value and precise with coefficient of variation (CV) of 1.22. These results were compared with those obtained by reference methods, zero-order UV spectrophotometric method and reversed-phase high-performance liquid chromatography (HPLC) method. A reversed-phase C18 column with aqueous phosphate (0.01 M): acetonitrile: glacial acetic acid (59:40:1, v/v/v) (pH 3.12) mobile phase was used and UV detector was set to 252 nm.[37]
- Hohyun Kim et al developed sensitive and selective liquid chromatographic method coupled with tandem mass spectrometry (LC-MS/MS) for the quantification of Ambroxol in human plasma. Domperidone was used as an internal standard. The plasma samples extracted using diethyl ether under

basic condition. A centrifuged upper layer was then evaporated and reconstituted with 200 ml methanol. The reconstituted samples were injected into a C_{18} XTerra MS column (2.1× 30mm) with 3.5 µm particle size. The mobile phase was composed of 20 mM ammonium acetate in 90% acetonitrile (pH 8.8), with flow rate at 250 µl/min. The mass spectrometer was operated in positive ion mode using turbo electrospray ionization. Nitrogen was used as the nebulizer, curtain, collision, and auxiliary gases. Using MS/MS with multiple reaction monitoring (MRM) mode, Ambroxol was detected without severe interferences from plasma matrix. Ambroxol produced a protonated precursor ion ($[M+ H]^+$) at m/z 379 and a corresponding product ion at m/z 264. And internal standard (Domperidone) produced a protonated precursor ion ($[M+ H]^+$) at m/z 426 and a corresponding product ion at m/z 174. Detection of Ambroxol in human plasma was accurate and precise, with quantification limit at 0.2 ng/ml.[38]

- K. Vyas et al detected three unknown impurities in Loratadine bulk drug at levels below 0.1% by a simple isocratic reversed-phase high performance liquid chromatography (HPLC). These impurities were isolated from mother liquor sample of Loratadine using reversed-phase preparative HPLC. Based on the spectral data (IR, NMR and MS) the structures of these impurities were characterized as 11-(N-carboethoxy-4-piperidylidene)-6,11- dihydro-5H-benzo(5,6) cyclopenta(1,2-b)-pyridine (I), 8-bromo-11-(N-carboethoxy-4-piperidylidene)-6,11-dihydro-5Hbenzo(5,6) cyclopenta (1,2-b)-pyridine (II) and 8-chloro-11-(N-carboethoxy-4-piperidylidene)-5H-benzo(5,6) cyclopenta (1,2-b)-pyridine (III).[39]

- Ophelia Q.P. Yin et al developed a high-performance liquid chromatographic (HPLC) method with fluorescence detection for the simultaneous determination of Loratadine (L) and its metabolite, Descarboethoxyloratadine (DCL), in human plasma. The linearity for L and DCL was within the concentration range of 0.5–16 ng/ml. The coefficient of variation of intra- and inter-day assay was <8.3%, with accuracy ranging from 98.3 to 105.7%. The lower limit of quantification was 0.5 ng/ml for both L and DCL.[40]
- Satyanarayana et al studied high performance liquid chromatography (HPLC) and second derivative spectrophotometry for the simultaneous determination of Montelukast and Loratadine in pharmaceutical formulations, HPLC separation was achieved with a Symmetry C18 column and sodium phosphate buffer (pH adjusted to 3.7): acetonitrile (20:80, v/v) as eluent, at a flow rate of 1.0 ml/min. UV detection was performed at 225 nm. 5-Methyl 2-nitrophenol was used as internal standard for the purpose of quantification of both the drugs in HPLC. In the second-order derivative spectrophotometry, for the determination of Loratadine the zero-crossing technique was applied at 276.1 nm, and for Montelukast peak amplitude at 359.7 nm (tangent method) was used. Both methods were fully validated and comparison was made for assay determination of selected drugs in formulations.[41]
- N.A. El Ragehy et al suggested four stability-indicating procedures have been suggested for determination of the non sedating antihistaminic agent Loratadine. Loratadine being an ester undergoes alkaline hydrolysis and the corresponding acid derivative was produced as a degradation product. Its identity was confirmed using IR and MS. The first procedure was based on determination of Loratadine by HPLC with detection at wavelength, 250 nm.

Mobile phase was acetonitrile: orthophosphoric acid (35:65) and benzophenone was used as an internal standard. Sensitivity range was 5.00–50.00 µg/ml. Second determination was a densitometric procedure based on determination of Loratadine in the presence of its degradate at 246 nm using the mobile phase; Methanol: Ammonia (10:0.15). Sensitivity range was 1.25–7.50 µg/spot. The third procedure was a spectrophotometric method where a mixture of Loratadine and its degradate are resolved by first derivative ratio spectra. Sensitivity range was found to be 3.00–22.00 µg/ml, upon carrying out the measurements at wavelengths 236, 262.4 and 293.2 nm. The fourth procedure was based on second derivative spectrophotometry, where D2 measurements are carried out at 266 nm. The sensitivity range was 3.00–22.00 µg/ml.[42]

- Coral Barbas b and Maarit Heinanena was described for Ambroxol, *trans*-4-(2-amino-3,5-dibromobenzylamino) cyclohexanol hydrochloride, and benzoic acid separation by HPLC with UV detection at 247 nm in a syrup as pharmaceutical preparation. Optimal conditions were: Column Symmetry Shield RPC8, 5 mm 250×4.6 µm, and methanol:(H_3PO_4 8.5 mM/triethylamine pH 2.8) 40:60 v/v.[43]
- John E. Koundourellis et al described method for Ambroxol in the presence of different preservatives in pharmaceutical formulations. The method was separates used to Ambroxol from methyl- ethyl-, propyl- and butyl paraben and from other multi-component mixtures. The retention behaviour of Ambroxol and parabens as a function of both pH and mobile phase composition was investigated. The eluents were monitored with a UV detector at 247nm.[44]

- C. Barbas et al developed and validated a HPLC method employing a Symmetry Shield RP8 column for loratadine and related compounds measurement. The mobile phase consisted of methanol-buffer A (65:35, v/v), being buffer A: H3PO4 10 mM (H2O) brought up to pH 7.00 with triethylamine. UV detection was performed at 244 nm. Validation parameters for linearity, accuracy and precision are in agreement with ICH guidelines for all the analytes and that permits to consider the method reliable and suitable for application to long-term stability and purity studies.[45]
- Grzegorz Bazylak and Luc J. Nagels developed analytical method for Simultaneous high-throughput determination of Clenbuterol, Ambroxol and Bromhexine in pharmaceutical formulations by isocratic HPLC system with potentiometric detection, A silica column (250× 4.6 mm i.d.) was used as a stationary phase with acetonitrile (ACN)-ethanol-perchloric acid (1.66 mM) (60:2:38, v/v/v) (pH 2.45) as mobile phase and RP18 hybrid silica polymer column eluted with Acetonitrile-phosphoric acid (20 mM) (25:75, v/v) (pH 2.60).[46]
- Taijun Hang et al developed a rapid, simple and sensitive LC–MS/MS method for simultaneous determination of amoxicillin and ambroxol in human plasma using clenbuterol as internal standard (IS), was performed using electrospray ionization. The plasma samples were subjected to a simple protein precipitation with methanol. Separation was achieved on a Lichrospher C18 column (150mm×4.6mm ID, dp 5µm) using methanol (containing 0.2% of formic acid) and water (containing 0.2% of formic acid) as a mobile phase by gradient elution at a flow rate of 1.0 ml/min. Detection was performed using electrospray ionization in positive ion multiple reaction monitoring (MRM)

mode by monitoring the ion transitions from *m/z* 365.9→348.9 (Amoxicillin), *m/z* 378.9→263.6 (Ambroxol) and *m/z* 277.0→203.0 (IS). Calibration curves were linear in the concentration range of 5–20,000 ng/ml for Amoxicillin, and 1–200 ng/ml for Ambroxol, with the intra- and inter-run precisions of <9% and the accuracies of 100±7%.[47]

- M. Pospısilova et al determined expectorant drugs Ambroxol (AX) and Bromhexine (BX) by capillary isotachophoresis (ITP) with conductimetric detection. The leading electrolyte (LE) was a buffer solution that contained 5 mM picolinic acid and 5 mM potassium picolinate (pH 5.2). The terminating electrolyte (TE) was 10 mM formic acid. The driving current was 80 mA (for:200 s) or 50 mA (for:350 s) and the detection current was 20 mA. The effective mobilities of AX and BX (evaluated with tetraethylammonium as the mobility standard) were 18.8×10^{-9} m^2 V^{-1} s^{-1} and 14.3×10^{-9} m^2 V^{-1} s^{-1} respectively.[48]
- Li Huande et al described a rapid, sensitive and specific method for determination of Ambroxol in human plasma using high performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC–MS/ESI), Ambroxol and the internal standard (IS), fentanyl, were extracted from plasma by *N*-hexane-diethyl ether (1:1, v/v) after alkalinizeation with ammonia water. A centrifuged upper layer was then evaporated and reconstituted with 100 µl mobile phase. Chromatographic separation was performed on a BDS HYPERSIL C18 column (250mm×4.6 mm, 5.0 µm, Thermoelectron corporation, USA) with the mobile phase consisting of 30mM ammonium acetate (0.4% formic acid)–acetonitrile

(64:36, v/v) at a flow-rate of 1.2 ml/min, Detection and quantitation was performed by the mass spectrometer.[49]

- E. Satana et al described a flow-injection UV spectrophotometric method for the determination of Ambroxol Hydrochloride in tablets. The quantitative determination of Ambroxol was performed at 245 nm using distilled water as the carrier solvent. In this study, the flow rate, loop volume, and the number of injections per hour were 15 ml/min, 193µl, and 100, respectively. The analytical signal of Ambroxol was linear in the concentration range of 40–200 µg/ml. The detection limit and limit of quantification were found as 11.55 and 38.49µg/ml, respectively. A relatively high recovery value (100.4%) shows the accuracy of the proposed method.[50]
- Weng Naidong et al developed and validated for the simultaneous analysis of antihistamine drug loratadine (LOR) and its active metabolite descarboethoxy-loratadine (DCL) in human plasma using liquid chromatography with tandem mass spectrometric detection (LC/MS/MS), Deuterated analytes, i.e. LOR-d3 and DCL-d3 were used as the internal standards (I.S.). Analytes were extracted from alkalized human plasma by liquid/liquid extraction using hexane. The extract was evaporated to dryness under nitrogen, reconstituted with 0.1% (v/v) of trifluoroacetic acid (TFA) in acetonitrile, and injected onto a 50× 3.0 mm i.d. 5 µm, silica column with an aqueous-organic mobile phase consisted of acetonitrile, water, and TFA (90:10:0.1, v/v/v). The chromatographic run time was 3.0 min per injection and flow rate was 0.5 ml/min. The retention time was 1.2 and 2.0 min for LOR and DCL, respectively.[51]

- Isam Ismail Salem et al worked and developed a sensitive and specific liquid chromatography electrospray ionization ion-trap mass spectrometry (LC–ESI-IT-MS/MS) method for the identification and quantitation of Loratadine in human plasma, After the addition of the internal standard (IS), plasma samples were extracted using isooctane: isoamyl alcohol mixture. The compounds were separated on a prepacked Zorbax phenyl column using a mixture of acetonitrile, 0.20% formic acid as mobile phase. A Finnigan LCQDUO ion-trap mass spectrometer connected to a Waters Alliance high performance liquid chromatography (HPLC) was used, The method was proved to be sensitive and specific by testing six different plasma batches.[52]
- G. Srinubabu et al developed a simple, sensitive and reliable method for simultaneous quantification of loratadine and desloratadine in human plasma. Author performed on-line coupling of extraction with Cyclone P 50mm×0.5mm 50µm HPLC column and chromatographic separation was performed with Zorbax XDB C18 50mm×2.1mm 5µm, followed by quantification with mass detector. The method was validated and showed good performances in terms of linearity, sensitivity, precision, accuracy and stability.[53]
- Abolhassan Ahmadiani et al developed a simple and rapid high-performance liquid chromatographic method with fluorescence detection for the determination of Loratadine in small volume of plasma samples. Liquid–liquid extraction of Loratadine and diazepam (as internal standard) from plasma samples was performed with *n*-butyl alcohol/*n*-hexane (2:98, v/v) in alkaline condition followed by back-extraction into diluted perchloric acid. Chromatography was carried out using a C8 column (250 × 4.6 mm, 5µm)

under isocratic elution with acetonitrile-20 mM sodium dihydrogen phosphate-triethylamine (43:57:0.02, v/v/v), pH 2.4. Analyses were run at a flow-rate of 1.0 ml/min at room temperature.[54]

- M.M. Mabrouk et al described highly sensitive, simple and accurate reversed phase liquid chromatographic and first derivative spectrophotometric methods for determination of antihistaminic drug loratadine [I] and nasal decongestant drug pseudoephedrine sulphate. The HPLC method involves separation of [I] and [II] on m-BondaPak C18 column using mixture of (methanol: H_2O: phosphoric acid: ammonium dihydrogen phosphate) (220:300:2:3 g) (V/V/V/W), 60 and 40% acetonitrile as mobile phase flowing at 2 ml/min with ultraviolet detection at 247 nm. The spectrophotometric method is based on recording the first derivative spectra for [I] and [II] at 307, 266 nm, respectively, of their solutions in 0.1M hydrochloric acid using the acid as blank.[55]

4. METHODOLOGY

4. SECTIONS OF METHODOLOGY

4.1 APPARATUS AND EQUPIMENTS

4.2 MATERIAL AND REAGENTS

4.3 PREPARATION OF SOLUTIONS FOR HPLC

4.4 RP-HPLC METHOD DEVELOPMENT AND OPTIMIZATION

4.5 METHOD VALIDATION

4.6 FORCED DEGRADATION STUDY

4.1 APPARATUS AND EQUIPMENTS:

- Shimadzu HPLC system

Table 4.1 HPLC system specification

Name	Specifications
Pump	LC-20 AT solvent delivery system
Detector	SPD-20A UV-Visible detector
Data processor	Spinchrome CFR version 2.4.1.93
Column	Phenomenex C8 (5 µm, 250 mm × 4.6 mm i.d.)
Rheodyne injector	20 µl loop

Manufacturer: Shimadzu, Japan

- Analytical Balance (Max. 200 gm-Min. 0.0001 mg)

Model: TE 2145

Manufacturer: Sartorius, India.

- Analytical Balance (Max. 300 gm-Min. 0.01 gm)

Model: DS-852J SERIES

Manufacturer: ESSAE, Electronic Weighting Scale, India

- A double beam UV-Visible spectrophotometer having two matched cells with 1cm light path

Model: Pharmspec-1700

Manufacturer: Shimadzu, Japan

- pH Meter

Model: 7007

Manufacturer: Digisun electronics

- Hot air oven

  Model: PSM 03

  Manufacturer: Thermoelectrical co.

- Ultrasonicator

  Model: RC-SYSTEM MU-1700

  Supplier: Servewell instruments.

- Distillation Apparatus

  Model: VQMD 2.5L

  Manufacturer: Srinivash products

4.2 REAGENTS AND MATERIALS:

4.2.1 Materials:

- Ambroxol Hydrochloride (AMH) and caffeine (CAF) - Working standard grade was supplied by Jugat Pharmaceutical Pvt. Ltd. (Bangalore, India) and its claimed purity was 99.92% and 99.82% respectively.
- Loratadine (LOR) – working standard grade was supplied by Microlabs Ltd. (Bangalore, India) and its claimed purity was 99.86%.

4.2.2 Reagents:

- Water (HPLC grade)
- Methanol (HPLC grade), Spectrochem
- Orthophosphoric Acid (HPLC grade), Merck, INDIA
- Triethylamine (HPLC grade), Merck, INDIA
- Hydrochloric acid (35% GR), Merck, INDIA
- Sodium hydroxide, Merck, INDIA
- Hydrogen peroxide, Merck, INDIA

- Sodium bisulphite, Sigma Aldrich, INDIA

4.3 PREPARATION OF SOLUTIONS FOR HPLC:

4.3.1 Stock solution for AMH (1000μg/ml):

An accurately weighed quantity of AMH working/reference standard about 50 mg was transferred into 50 ml volumetric flask and made up to mark with methanol (1000μg/ml).

4.3.2 Stock solution of LOR (100μg/ml)

An accurately weighed quantity of LOR working/reference standard about 50 mg was transferred into 50 ml volumetric flask and made up to mark with methanol (1000μg/ml). About 1ml of this solution was transferred to 10 ml volumetric flask and volume was made to mark with methanol (100μg/ml).

4.3.3 Combined stock solution I of AMH (1000 μg/ml) and LOR(100 μg/ml)

An accurately weighed quantity of AMH working/reference standard about 50 mg and LOR working/reference standard about 5 mg was transferred in 50ml volumetric flask and made up to mark with methanol.

4.3.4 Combined stock solution II of AMH (100 μg/ml) and LOR (10 μg/ml)

About 1ml of combined stock solution I was transferred to 10 ml volumetric flask and volume was made to mark with methanol.

4.3.5 Stock solution for CAF (100 μg/ml)

An accurately weighed quantity of CAF working/reference standard about 50 mg was transferred into 50 ml volumetric flask. About 25 ml of water was

added and sonicated to dissolve. The solution was cooled to the room temperature and made up to mark with water. 5 ml of this solution was transferred to 50 ml volumetric flask and made up to mark with water to get concentration of 100 µg/ml.

4.3.6 Standard preparation

For calibration curve series of dilution of drugs were prepared by transferring 1, 2, 3, 4 and 5 ml aliquots of combine standard stock solution II and 1, 2 ml aliquots of combined stock solution I separately in 10 ml volumetric flasks along with 1ml stock solution of CAF and volume made up to mark with mobile phase.

4.3.7 Sample preparation (50 µg/ml):

10 tablets were accurately weighed and crushed. Accurately weighed about 175.8 mg powder which contains 60 mg of AMH and 5 mg of LOR was transferred into 50 ml volumetric flask. About 25 ml of methanol was added and sonicated for 10 minutes. The solution was cooled to the room temperature and made up to volume with methanol. The solution was filtered through whatman filter paper (grade 41); filtrate was collected after discarding first few ml. 1 ml of this filtrate and 1 ml of stock solution of CAF (100 µg/ml) were transferred to 10 ml volumetric flask and diluted to 10 ml with mobile phase. Above solution was injected into HPLC system. Peak areas were recorded for all the peaks. The amount of AMH and LOR present in the tablets were calculated using single point analysis by following equation.

$$C_1/C_2 = R_1/R_2$$

Where, C_1 = Concentration of Sample Solution

C_2 = Concentration of Standard Solution

R_1 = Peak Area Ratio of Drug to Internal Standard for Sample Solution

R_2 = Peak Area Ratio of Drug to Internal Standard for Standard Solution

4.3.8 Mobile phase preparation:

1 ml orthophosphoric acid was diluted to 1000 ml with double distilled water to get concentration of 0.1% OPA. About 300 ml of OPA was mixed with 700 ml of methanol and pH was adjusted to 5.0 with 0.1% triethylamine solution in methanol. Then resulting solution was filtered through 0.22 µ Supor 200 membrane filter and degassed in sonicator for 20 minutes. This solution was used as mobile phase.

4.4 RP-HPLC Method development and optimization:

The standard solution of AMH, LOR and CAF were studied on HPLC system using different mobile phase composition and column to determine AMH and LOR in presence of degraded products. Degraded samples were prepared by systematic forced degradation study. These samples were used for method development trials to optimize the method as a stability indicating method.

4.4.1 Selection of detection wavelength:

The standard solutions of 10 µg/ml of AMH, LOR and CAF in methanol were scanned over the range of 190 nm to 400 nm wavelengths. The common wavelength of absorption was found to be 255 nm. So the wavelength selected for the determination of AMH and LOR was 255nm.

4.4.2 Selection of mobile phase

Optimization can be started only after a reasonable chromatogram has been obtained. A reasonable chromatogram means that more or less symmetrical peaks on the chromatogram detect all the compounds. By slight change of the mobile phase composition, the position of the peaks can be predicted within the range of investigated changes. An optimized chromatogram was the one in which all the peaks are symmetrical and well separated in less run time.

The mobile phase was selected on the basis of best separation, peak purity index, peak symmetry, theoretical plate etc. So, numbers of trial were taken for the selection of mobile phase as shown in Table 5.1/5.2/5.3. After number of trial Methanol: 0.1% OPA (70:30% v/v, pH adjusted to 5.0 with triethylamine solution) was selected.

4.4.3 Selection of pH:

The pH of the mobile phase was adjusted between 2.0 to 8.0 in different mobile phase and chromatogram was study for peak tailing, resolution between degraded product. So pH of mobile phase was adjusted to 5.0 with 0.1% triethylamine solution (HPLC grade).

4.4.4 Selection of flow rate

Different mobile phase flow rates (0.7, 1.0, 1.5ml/min) were investigated. The optimum flow rate for which the column plate number (N) was maximum, with the best resolution between all components and with a short run time (20min) was selected

4.4.5 Selection of Internal Standard (IS)

The selection of IS was made on the basis of stability with drug, asymmetric factor, resolution and number of theoretical plates. Caffeine (10µg/ml) was studied to use as IS for the chromatographic procedure in different mobile phase composition having different pH.

4.4.6 Finalized Chromatographic conditions:

The finalized HPLC system specifications are shown in below table.

Table4.2 Finalised HPLC system specification

Parameters	Specifications
Column	Phenonemex C_8 (250×4.6mm, 5µm)
Mobile phase	Methanol: 0.1%OPA (70:30%v/v)
pH of mobile phase	5.0 using triethylamine solution (0.1%)
IS	CAF(10µg/ml)
Flow rate(ml/min)	1.5 ml/min
Detection wavelength	255nm
AUFS	0.1000
Pressure	18.9 Mpa

4.5 METHOD VALIDATION

Validation of RP-HPLC Method

4.5.1 Accuracy

To study the accuracy, 10 tablets were weighed and powered. Analysis of the same was carried out as mentioned in section 4.3.7. Recovery studies were carried out by standard addition method by adding the known amount of AMH and LOR (reference standard) separately to the preanalyzed sample at three different concentration levels i.e. 80%, 100%, and 120% of assay concentration and percent recoveries were calculated.

From the above filtrate about 1 ml of filtrate of sample was pipetted out and transferred to three 10 ml volumetric flasks separately along with this 0.96, 1.20, 1.44 ml of aliquot from the combine stock solution I and 1 ml aliquot from standard caffeine solution. All the solutions were filtered through 0.45 µm cellulose acetate filter and injected into HPLC system. Peak areas were recorded for all the peaks. Peak areas ratios between AMH to CAF and LOR to CAF were calculated. From the above data percent recoveries were calculated and the accuracy study was carried out for HPLC method.

4.5.2 Precision

The precision of an analytical method was studied by performing intra-day and inter-day precision.

4.5.2.1 Intra-day Precision

Variation of results within the same day was analyzed. Intra-day precision was determined by analyzing the combined standard solutions of Ambroxol hydrochloride (20, 40, 100µg/ml) and Loratadine (2, 4, 10µg/ml) with 10 µg/ml of caffeine in linearity range at three different time intervals on same day.

4.5.2.2 Inter-day Precision

Variation of results between the days was analyzed. Inter-day precision was determined by analyzing the combined standard solutions of Ambroxol hydrochloride (30, 50, 100µg/ml) and Loratadine (3, 5, 10µg/ml) with 10 µg/ml of caffeine in linearity range at three consecutive days.

4.5.3 Repeatability

Combined standard solutions of AMH (50µg/ml) and LOR (5µg/ml) with 10 µg/ml of CAF were prepared and analyzed. The solutions were analyzed six times and the standard deviation was calculated.

4.5.4 Reproducibility

Combined standard solutions of AMH (100 µg/ml) and LOR (10 µg/ml) with 10 µg/ml of CAF were prepared and analyzed. Both the solutions were prepared and analyzed by Analyst 1 and Analyst 2, separately. The values obtained were evaluated using F-test and t-test to verify their reproducibility.

4.5.5 Linearity and Range

The concentration ranges of 10-200 µg/ml for AMH and 1-20 µg/ml for LOR were prepared and analyzed. From the data, linearity and range were determined.

4.5.6 Limit of Detection and Limit of Quantitation

Detection limit and quantitation limit were determined based on the standard deviation of y – intercepts of six calibration curves and average slope of six calibration curves.

$$\text{LOD} = 3.3 \times \frac{\text{Standard Deviation of y} - \text{Intercepts of Six Calibration Curves}}{\text{Average Slope of Six Calibration Curves}}$$

$$\text{LOQ} = 10 \times \frac{\text{Standard Deviation of y} - \text{Intercepts of Six Calibration Curves}}{\text{Average Slope of Six Calibration Curves}}$$

4.5.7 System Suitability

Combined standard solutions of AMH (100 µg/ml) and LOR (10 µg/ml) with CAF (10 µg/ml) were prepared and analyzed six times. Chromatograms were studied for different parameters such as tailing factor, resolution and theoretical plates to see that whether they complies with the recommended limit or out of recommended limit.

4.5.8 Robustness

The effect of change in the pH of mobile phase and flow rate on the retention time, tailing factor, theoretical plates and resolution were studied. Combined standard solutions of AMH (100µg/ml), LOR (10 µg/ml) with CAF (10µg/ml) were prepared and analyzed at different pH (4.85, 5.00, 5.15) of the mobile phase and at different flow rate (1.45, 1.50, 1.54 ml/min).

4.6 FORCED DEGRADATION STUDY:

In order to establish whether the analytical method for the assay was stability indicating, tablets, pure active pharmaceutical ingredient (API) of AMH and LOR were subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid/base hydrolysis, oxidation, reduction and neutral in accordance with ICH Q1A (R2) guideline. Several trials with different severity of each stressed conditions were carried out to achieve 10-30% degradation of drugs.

4.6.1 Forced degradation studies of mixture of standard drugs :

- **For acid degradation:**

Standard of AMH (600mg) and LOR (50mg) was accurately weighed and transferred into three sets of 250 ml round bottom flasks. About 20 ml of HCl of different strengths (0.1N, 1.0N, 2N) was added to all flasks and refluxed on heated mantle for 6 hr at 60 ºC.

- **For basic degradation:**

Standard of AMH (600mg) and LOR (50mg) was accurately weighed and transferred into three sets of 250 ml round bottom flasks. About 20 ml of NaOH

of different strengths (0.1N, 0.5N, 0.75N, 1.0N) was added to all flasks and refluxed on heated mantle for 6 hr at 60 ºC.

- **For peroxide degradation:**

Standard of AMH (600mg) and LOR (50mg) was accurately weighed and transferred into three sets of 250 ml round bottom flasks. About 20 ml of H_2O_2 of different strengths (3%, 6% v/v) was added to all flasks and refluxed on heated mantle for 1 hr at 60 ºC.

- **For reduction degradation:**

Standard of AMH (600mg) and LOR (50mg) was accurately weighed and transferred into three sets of 250 ml round bottom flasks. About 20 ml of $NaHSO_3$ of different strengths (10%, 15% v/v) was added to all flasks and refluxed on heated mantle for 5hr at 60 ºC.

- **For neutral degradation:**

Standard of AMH (600mg) and LOR (50mg) was accurately weighed and transferred into three sets of 250 ml round bottom flasks. About 20 ml of water was added to all flasks and refluxed on heated mantle for 5 hr at 60 ºC.

About1ml of samples were withdrawn during the degradation study at different period of time (15min, 30min, 45min, 1hr, 2hr, 3hr, 4hr, 5hr). About 0.1ml of sample was taken into 10 ml volumetric flask in a few ml of mobile phase and sonicated for 10 minutes, This solution was cooled to the room temperature and

made up to volume with mobile phase. It was filtered through 0.45 µ cellulose acetate filter and filtrate was used for chromatographic analysis. .

Degradation conditions were optimized for API by study the % degradation of drug (10-30%) and same condition were applied for degradation of tables.

4.6.2Forced degradation studies of tablets:

- **For acid degradation:**

Twenty tablets were weighed, crushed and powder equivalent to 600 mg of AMH and 50 mg of LOR was added in three sets of 250 ml round bottom flasks. About 20 ml of 1N HCL was added to each flask and refluxed on heated mantle for 45min at 60 ºC.

- **For basic degradation:**

Twenty tablets were weighed, crushed and powder equivalent to 600 mg of AMH and 50 mg of LOR was added in three sets of 250 ml round bottom flasks. About 20 ml of 0.5N NaOH was added to each flask and refluxed on heated mantle for 1 hr at 60 ºC.

- **For peroxide degradation:**

Twenty tablets were weighed, crushed and powder equivalent to 600 mg of AMH and 50 mg of LOR was added in three sets of 250 ml round bottom flasks. About 20 ml of 3% H_2O_2 was added to each flask and refluxed on heated mantle for 30 min at 60 ºC.

- **For reduction degradation:**

Twenty tablets were weighed, crushed and powder equivalent to 600 mg of AMH and 50 mg of LOR was added in three sets of 250 ml round bottom flasks. About 20

ml of 10% $NaHSO_3$ was added to each flask and refluxed on heated mantle for 4 hr at 60 ºC.

- **For neutral degradation:**

Twenty tablets were weighed, crushed and powder equivalent to 600 mg of AMH and 50 mg of LOR was added in three sets of 250 ml round bottom flasks. About 20 ml of water was added to each flask and refluxed on heated mantle for 4 hr at 60 ºC.

About1ml of sample was withdrawn during the degradation study at time mentioned earlier. About 0.1ml of sample was taken into 10 ml volumetric flask in a few ml of mobile phase and sonicated for 10 minutes. This solution was cooled to the room temperature and made up to volume with mobile phase. It was filtered through 0.45 µ cellulose acetate filter and filtrate was used for chromatographic analysis.

5. RESULTS

5. RESULTS

5.1 REVERSE PHASE HIGH PERFORMANCE LIQUID CROMATOGRAPHY

5.1.1 SELECTION OF ANALYTICAL WAVELENGTH

5.1.2 OPTIMIZATION OF CHROMATOGRAPHIC CONDITION

5.1.3 CALIBRATION CURVE

5.1.4 ASSAY OF MARKETED FORMULATION

5.2 METHOD VALIDATION

5.2.1 ACCURACY

5.2.2 PRECISION

i. INRADAY

ii. INTREDAY

5.2.3 REPEATABILITY

5.2.4 REPRODUCIBLITY

5.2.5 SYSTEM SUITABILITY

5.2.6 ROBUSTNESS

5.3 FORCED DEGRADATION STUDY (API & Marketed formulation)

5.3.1 ACID DEGRADATION

5.3.2 BASE DEGRADATION

5.3.3 OXIDATIVE DEGRADATION

5.3.4 REDUCTIVE DEGRADATION

5.3.5 NEUTRAL DEGRATION

5.1.1 SELECTION OF ANALYTICAL WAVELENGTH

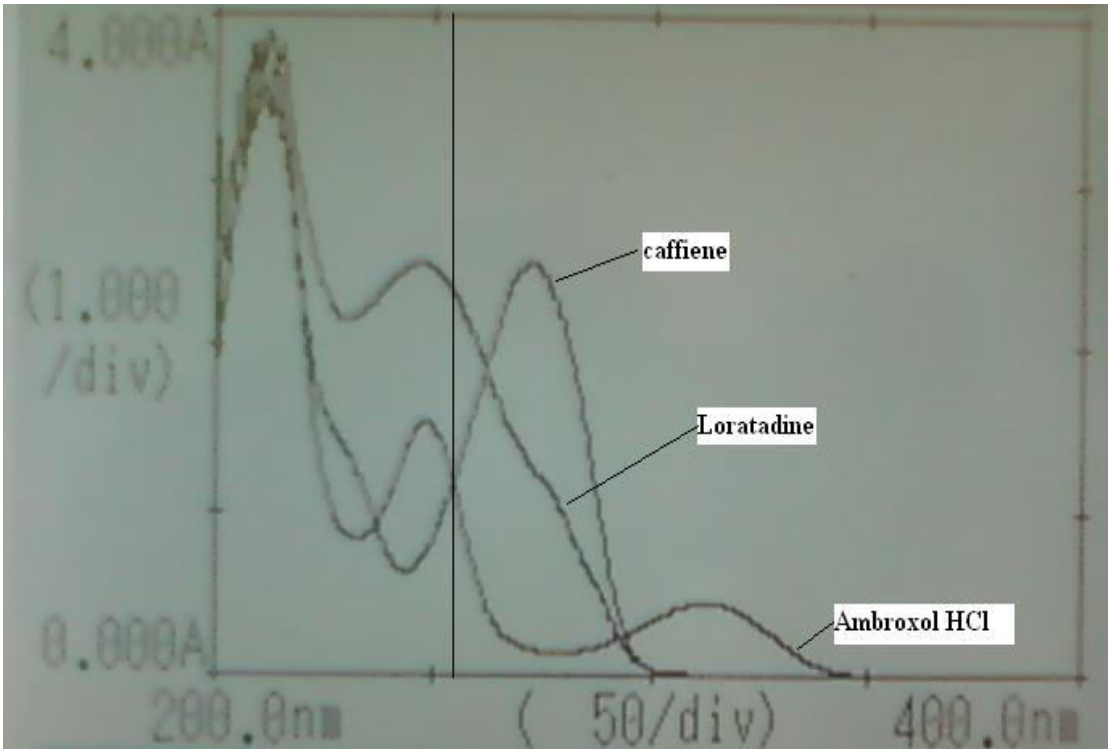


Figure: 5.1 Overlain Spectra of AMH, LOR and CAF for Selection of Analytical Wavelength in RP-HPLC Method

5.1.2 OPTIMIZATION OF CHROMATOGRAPHIC CONDITION

Table: 5.1 Results of Optimization of Chromatographic Condition

Mobile Phase Composition (ACN: AA) %v/v	pH	Column	Flow rate ml/min	Drug	R_t (min)	Tailing Factor	Theoretical Plates	Theoretical Plates/250 mm	Resolution
50:50	3	C_{18}	1	CAF	2.743	1.118	7753	31012	--
				AMH	3.030	0.510	2595	10380	1.586
				LOR	3.573	1.476	8121	32482	2.748
60:40	3	C_{18}	1	AMH	2.760	3.158	2111	4845	--
60:40	2	C_{18}	1	AMH	2.763	1.063	5223	20891	--
60:40	7	C_{18}	1	AMH	3.05	1.474	7324	29295	--
				CYPRO	4.0704	1.654	7145	28579	1.373
				LOR	10.167	1.386	11145	44581	21.159

Table: 5.2 Results of Optimization of Chromatographic Condition

Mobile Phase Composition (ACN: H_2O)% v/v	pH	Column	Flow rate ml/min	Drug	R_t (min)	Tailing Factor	Theoretical Plates	Theoretical Plates/ 250 mm	Resolution
60:40	2	C_{18}	1	LOR	3.473	1.840	4641	18565	--
60:40	2	C_{18}	1	CAF	2.690	1.097	4290	17160	--
				AMH	3.023	0.600	1507	4740	1.405
				LOR	3.370	1.905	5530	22119	1.411
60:40	6	C_{18}	1.2	CAF	2.550	1.722	4796	19184	--
				AMH	8.977	1.692	8439	33755	23.948
				LOR	16.723	0.867	2636	10544	9.172
60:40	6.4	C_{18}	1	CAF	3.217	1.300	7632	30527	--
				AMH	7.370	4.028	52	207	1.960
				LOR	11.703	2.167	9452	37807	1.896
60:40	7	C_{18}	1.2	CAF	2.540	1.500	6646	13292	--
				AMH	8.177	1.521	8959	17917	24.041
				LOR	19.793	2.997	190	7923	12.90

Table: 5.3 Results of Optimization of Chromatographic Condition

Mobile Phase Composition (MeOH:H_20) % v/v	pH	Column	Flow rate ml/min	Drug	R_t (min)	Tailing Factor	Theoretical Plates	Theoretical Plates/ 250 mm	Resolution
50:50	2.5	C_{18}	1	CAF	2.893	1.091	1199	4796	--
				AMH	4.093	1.892	3342	13367	4.008
				LOR	29.210	1.701	5836	23343	27.960
50:50	2	C_8	1	CAF	4.380	1.318	4505	18020	--
				AMH	4.610	1.500	5038	20152	1.517
				LOR	17.037	1.643	4343	17373	2.540
70:30	8	C_8	1	CAF	3.207	2.077	3050	12200	
				AMH	16.770	2.815	799	3195	10.438
				LOR	21.743	2.015	6464	25854	2.888
70:30	5	C_8	1	CAF	3.220	1.583	4747	18989	--
				AMH	3.670	1.607	4415	17661	2.212
				LOR	19.173	1.242	7830	31320	28.584
70:30	5	C_8	1.5	CAF	2.163	2.000	2586	10041	--
				AMH	2.517	1.957	2720	11567	1.968
				LOR	13.367	1.634	5134	20703	23.356

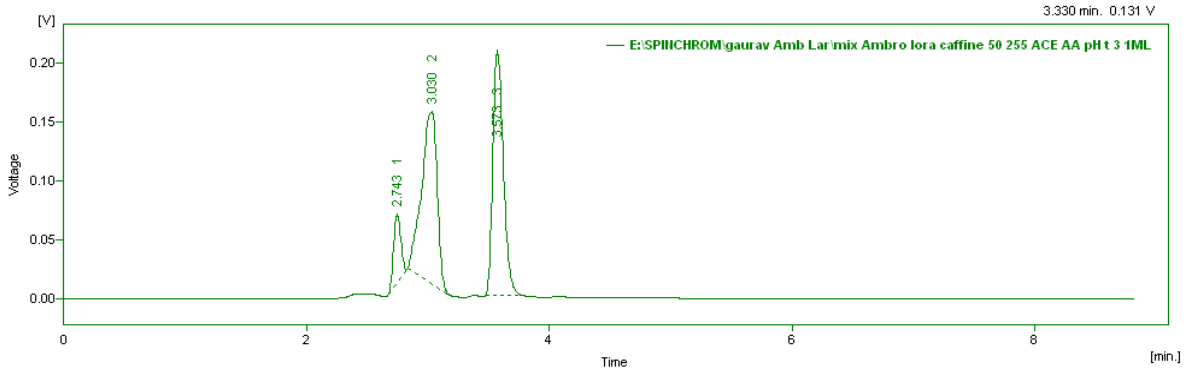


Figure: 5.2 Chromatogram of AMH, LOR and CAF in Acetonitrile: Ammonium Acetate (50:50 % v/v, pH 3.0) at Flow Rate 1 ml/min, at 255 nm on C_{18} column.

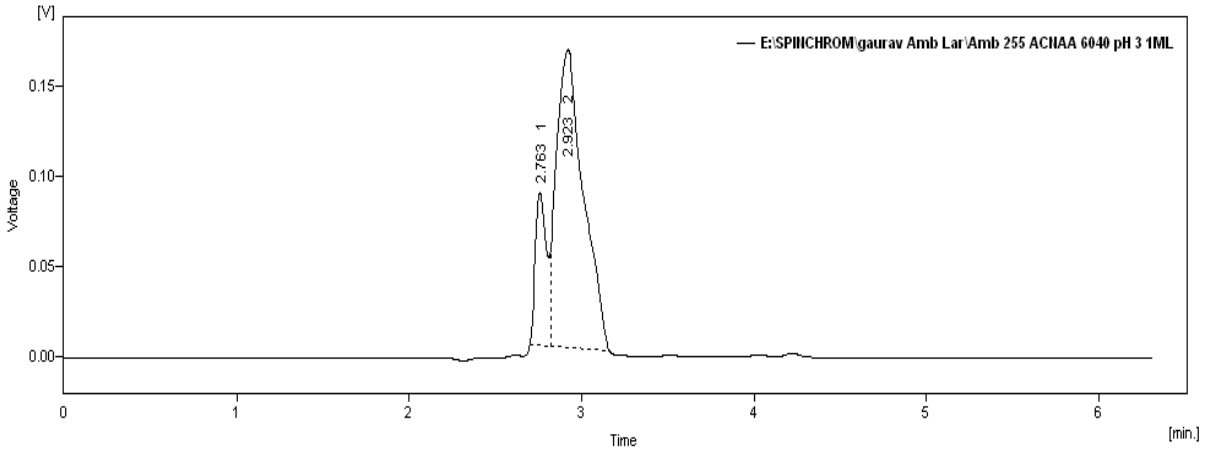


Figure: 5.3 Chromatogram of AMH in Acetonitrile: Ammonium Acetate (60:40 % v/v, pH 3.0) at Flow Rate 1 ml/min, at 255 nm on C_{18} column.

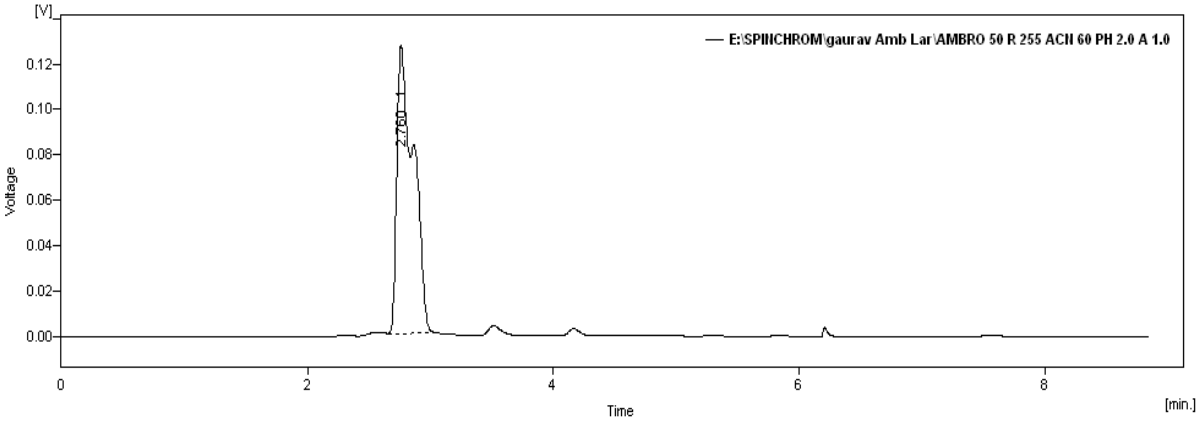


Figure: 5.4 Chromatogram of AMH in Acetonitrile: Ammonium Acetate (60:40 % v/v, pH 2.0) at Flow Rate 1 ml/min, at 255 nm on C_{18} column.

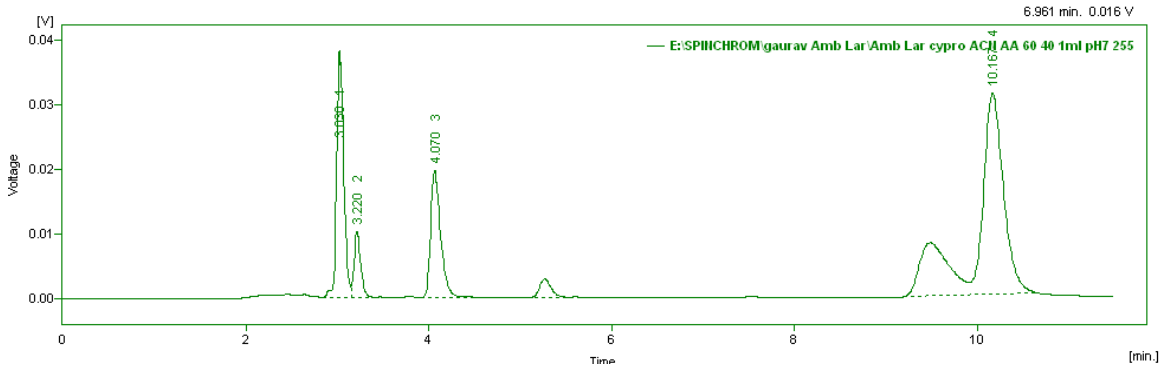


Figure: 5.5 Chromatogram of AMH, LOR and CYPRO in Acetonitrile: Ammonium Acetate (60:40 % v/v, pH 7.0) at Flow Rate 1 ml/min, at 255 nm on C_{18} column.

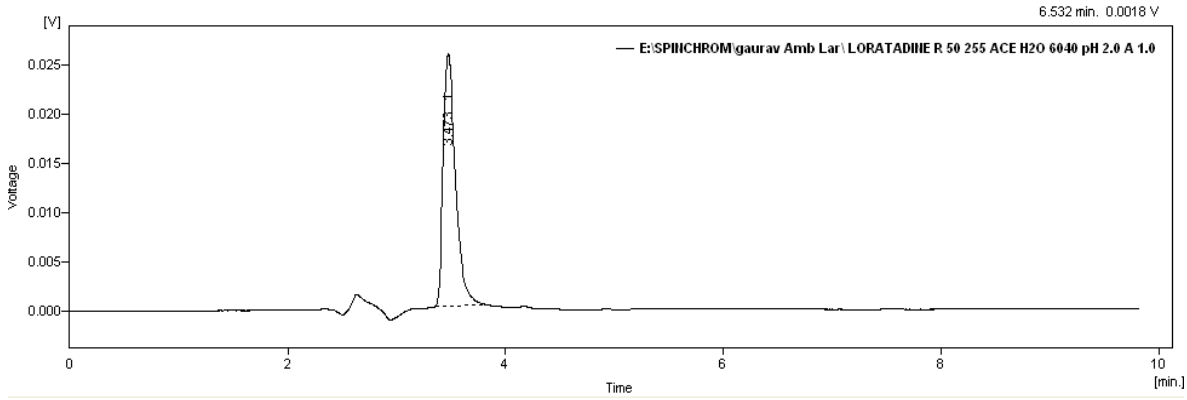


Figure: 5.6 Chromatogram of LOR in Acetonitrile: Water (60:40 %v/v, pH 2.0) at Flow Rate 1 ml/min, at 255 nm on C_{18} column.

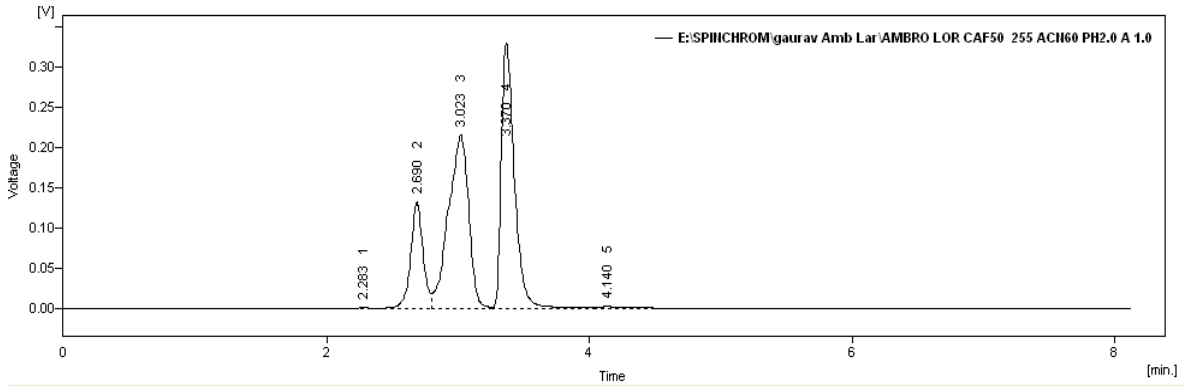


Figure: 5.7 Chromatogram of AMH, LOR and CAF in Acetonitrile: Water (60:40 % v/v, pH 2.0) at 1 ml/min Flow Rate, at 255 nm on C_{18} column.

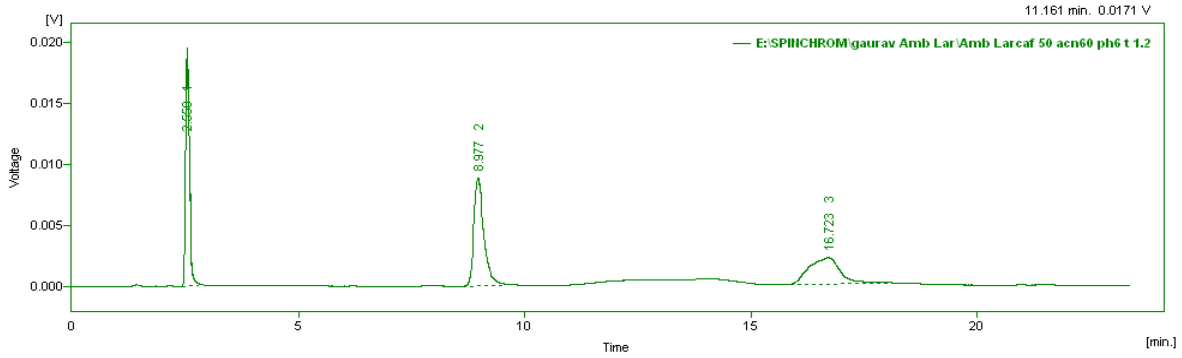


Figure: 5.8 Chromatogram of AMH, LOR and CAF in Acetonitrile: Water (60:40 % v/v, pH 6.0) at 1.2 ml/min Flow Rate, at 255 nm on C_{18} column.

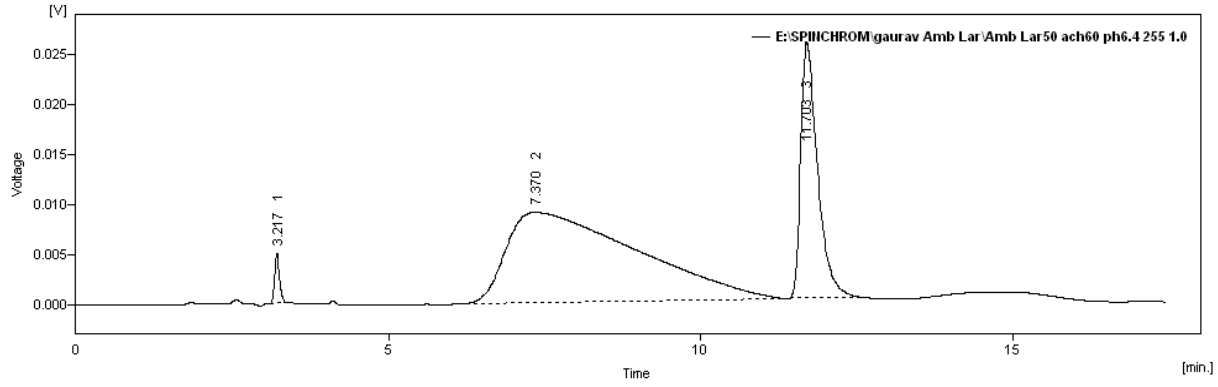


Figure: 5.9 Chromatogram of AMH, LOR and CAF in Acetonitrile: Water (60:40 % v/v, pH 6.4) at 1 ml/min Flow Rate, at 255 nm on C_{18} column.

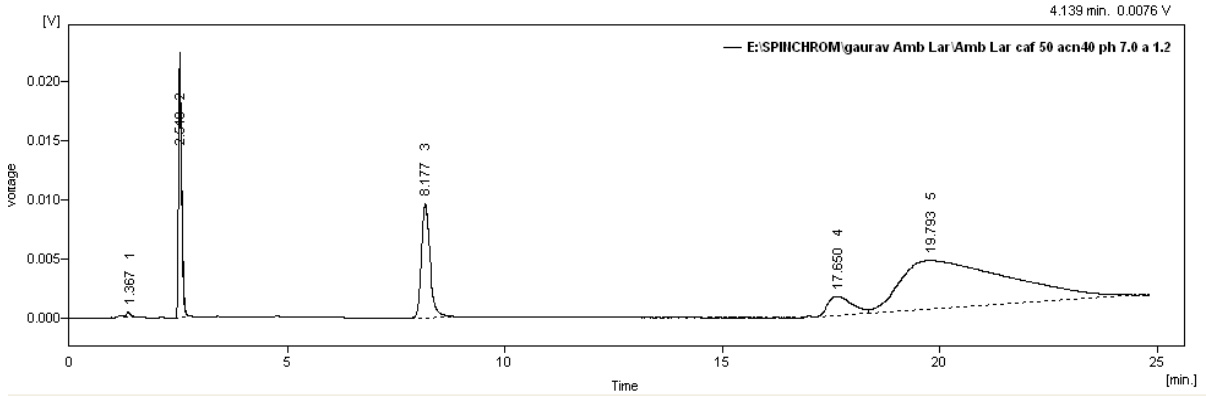


Figure: 5.10 Chromatogram of AMH, LOR and CAF in Acetonitrile: Water (60:40 % v/v, pH 7.0) at 1.2 ml/min Flow Rate, at 255 nm on C_{18} column.

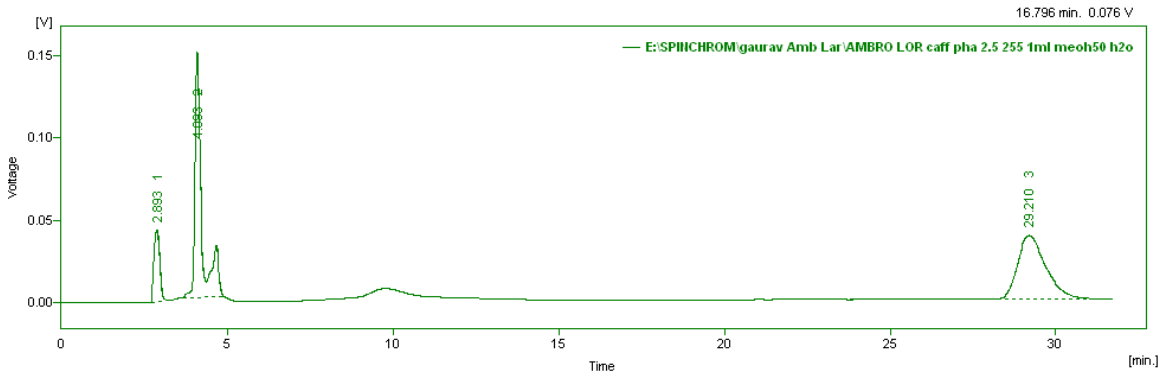


Figure: 5.11 Chromatogram of AMH, LOR and CAF in Methanol: Water (50:50 % v/v, pH 2.5) at Flow Rate 1ml/min, at 255 nm on C_{18} column.

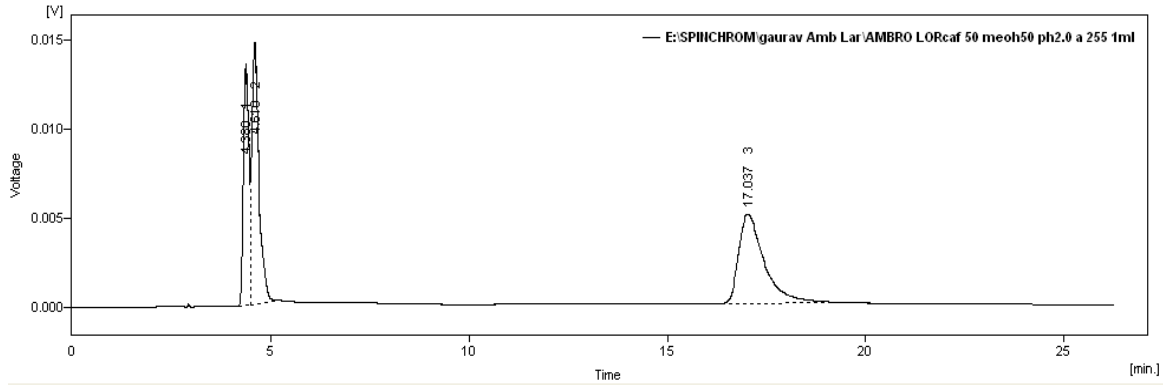


Figure: 5.12 Chromatogram of AMH, LOR and CAF in Methanol: Water (50:50 % v/v, pH 2.0) at Flow Rate 1ml/min, at 255 nm on C_{18} column.

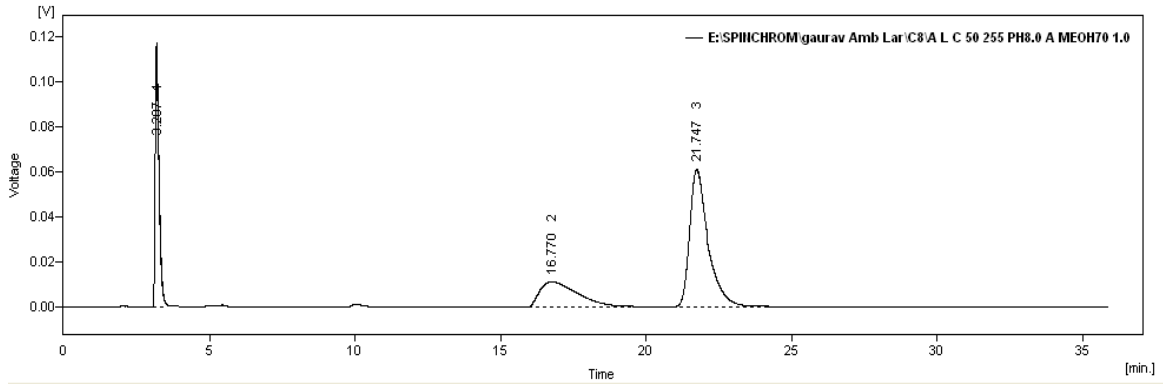


Figure: 5.13 Chromatogram of AMH, LOR and CAF in Methanol: Water (70:30 % v/v, pH 8.0) at Flow Rate 1ml/min, at 255 nm on C_{18} column.

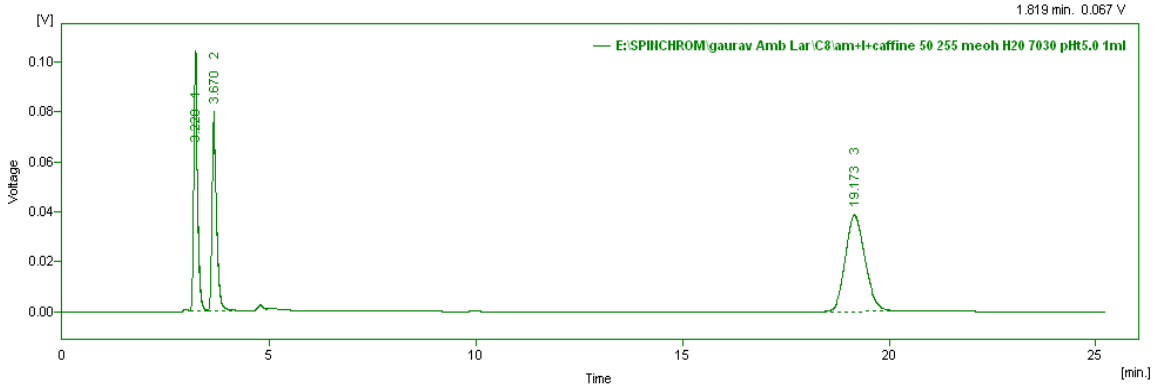


Figure: 5.14 Chromatogram of AMH, LOR and CAF in Methanol: Water (70:30 % v/v, pH 5.0) at Flow Rate 1ml/min, at 255 nm on C_{18} column.

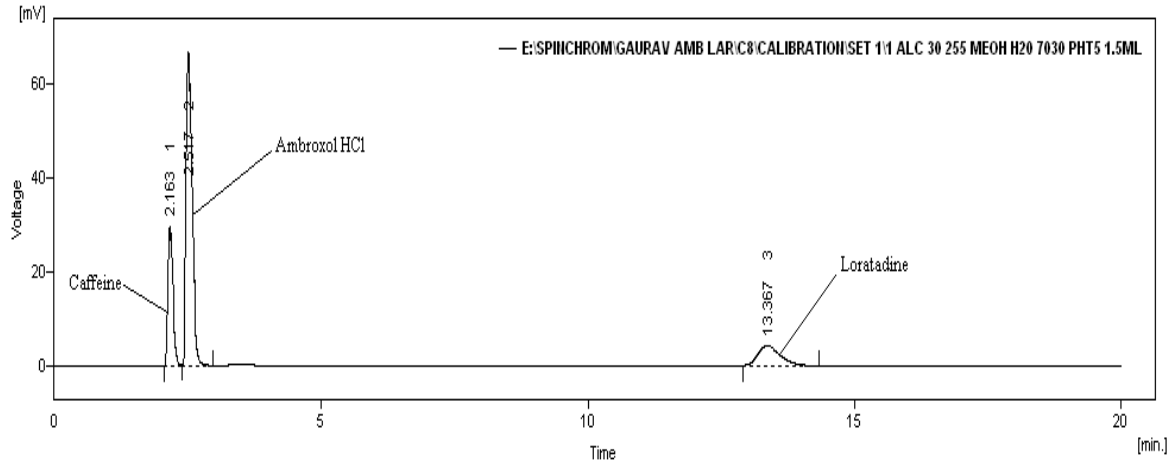


Figure: 5.15 Chromatogram of AMH, LOR and CAF in Methanol: Water (70:30 % v/v, pH 5.0) at Flow Rate 1.5ml/min, at 255 nm on C_{18} column.

5.1.2 CALIBRATION CURVE

Table: 5.4 Results of Calibration Curve of AMH

Concentration (µg/ml)	Peak Area Ratio							±SD	%RSD
	I	II	III	IV	V	VI	Mean		
10	0.7780	0.8900	0.7330	1.1970	1.2104	1.3007	1.0181	0.2466	4.0376
20	1.9800	2.000	1.8230	1.9820	1.9891	1.874	1.9413	0.0740	0.6357
30	2.6390	2.8160	2.3320	2.7350	2.6536	2.7617	2.6562	0.1722	1.0805
40	3.1400	4.1350	3.1360	3.500	3.3122	3.4425	3.4443	0.3702	1.7913
50	3.5380	4.8800	3.9640	4.500	4.5846	4.5507	4.3362	0.4911	1.8876
100	8.800	8.5580	8.900	8.320	7.3873	8.3226	8.3813	0.5423	1.0784
200	17.5300	16.2700	16.6900	16.3160	18.1204	17.2356	17.169	0.7206	0.8395

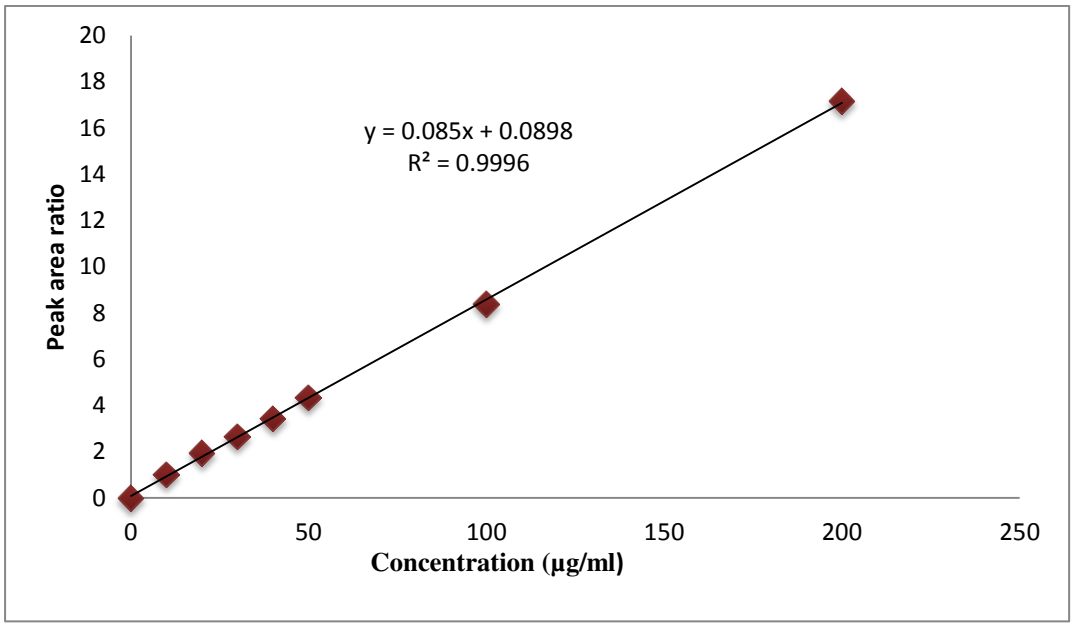


Fig: 5.16 Calibration Curve of AMH at 255 nm for RP-HPLC Method

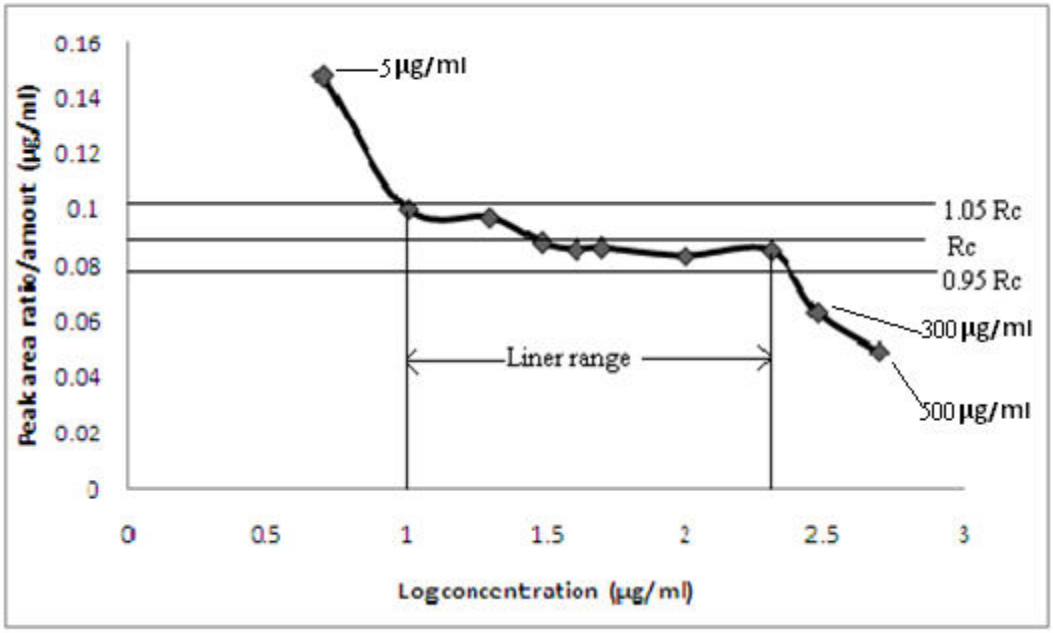


Fig: 5.17 Graph for Linearity Study of AMH

Table: 5.5. Linear Regression Analysis of Calibration Curves for AMH

Parameters	**AMH at 255 nm**
Linearity Range (µg/ml)	10-200
Slope	0.085
Intercept	0.0898
Correlation Coefficient (r^2)	0.9996
LOD (µg/ml)	2.2866
LOQ (µg/ml)	6.9299

Table: 5.6 Results of Calibration Curve of LOR

Concentration (µg/ml)	Peak Area Ratio							±SD	%RSD
	I	II	III	IV	V	VI	Mean*		
1	0.1740	0.4750	0.6320	0.7890	1.1400	2.0300	4.0300	0.0699	4.8489
2	0.2640	0.4600	0.5300	0.7460	1.1200	2.0270	4.1260	0.0725	2.6734
3	0.2100	0.4950	0.6370	0.7560	0.9010	1.9460	3.5580	0.0556	1.6272
4	0.2350	0.5100	0.5800	0.6710	0.8840	1.9210	4.0900	0.0711	1.6883
5	0.1920	0.4620	0.5069	0.6203	0.7823	1.8900	3.0765	0.1911	3.4920
10	0.3677	0.3093	0.5317	0.6296	0.6477	1.1595	2.8966	0.3327	3.0322
20	0.1740	0.4750	0.6320	0.7890	1.1400	2.0300	4.0300	0.5416	2.4872

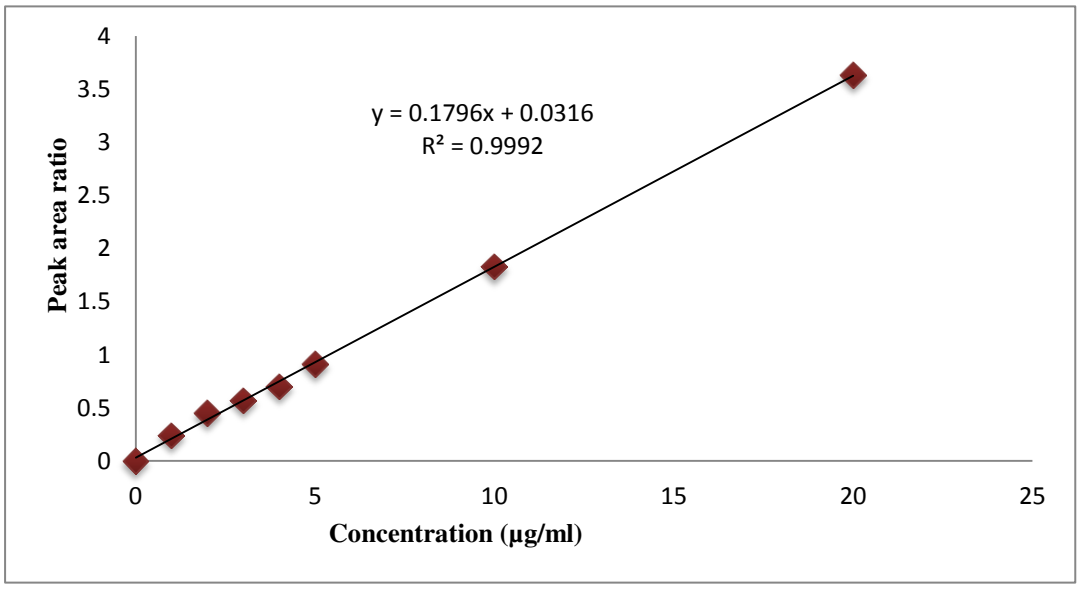


Fig: 5.18 Calibration Curve of LOR at 255 nm for RP-HPLC Method

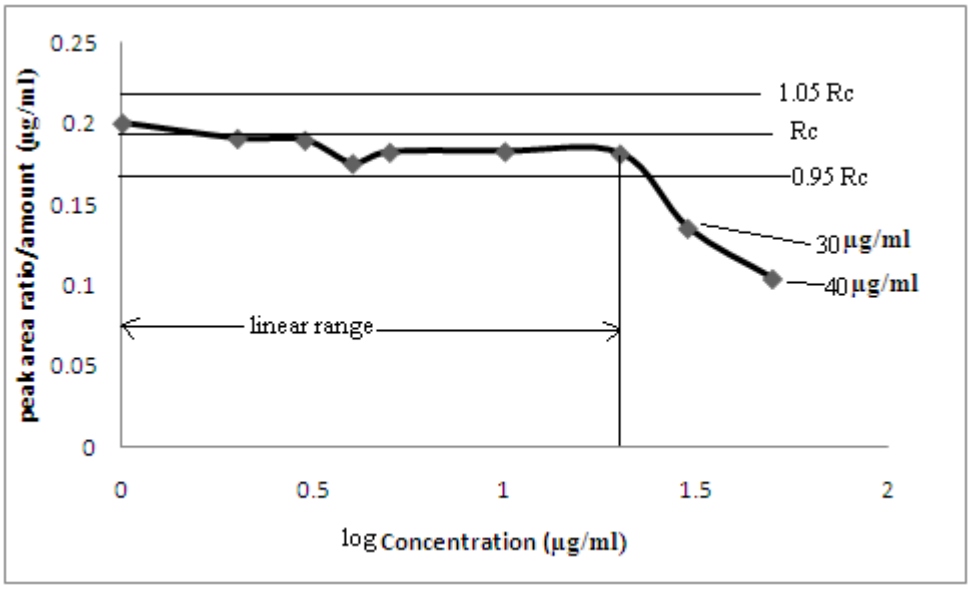


Fig: 5.19 Graph for Linearity Study of LOR

Table: 5.7 Linear Regression Analysis of Calibration Curves for LOR

Parameters	**LOR at 255 nm**
Linearity Range (µg/ml)	1-20
Slope	0.1796
Intercept	0.0316
Correlation Coefficient (r^2)	0.9992
LOD (µg/ml)	0.4530
LOQ (µg/ml)	0.8728

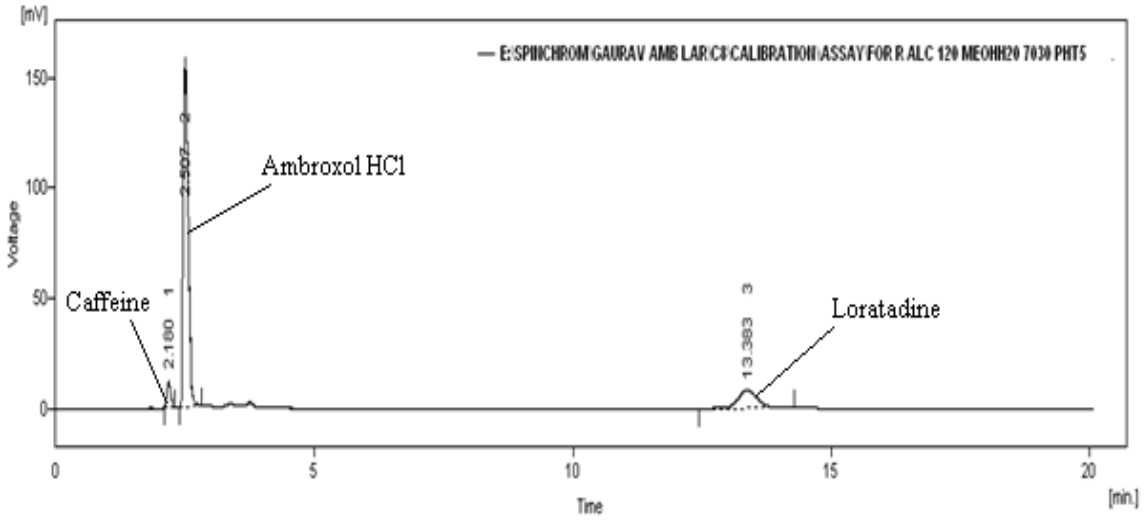


Fig.5.20 Assay of Tablet Formulation by RP-HPLC Method

Table: 5.8 Results of Chromatogram of Sample Solution

Analyte	Retention Time (min)	Area (mV.sec)	Tailing Factor (T)	Theoretical Plates (N)	Theoretical Plates /250 mm	Resolution (R)
CAF	2.183	14.836	1.500	2576	10217	--
AMH	2.523	148.902	1.478	2811	12890	1.987
LOR	13.497	22.011	1.311	5077	20684	23.198

5.1.3 ASSAY OF MARKETED FOMULATION

Table: 5.9 Assay Results of Tablet Formulation by RP-HPLC Method

Sr. No.	Amount Present(mg/tab)		Amount Found (mg/tab)		% Amount found	
	AMH	LOR	AMH	LOR	AMH	LOR
1	60	5	61.941	4.832	103.23	96.65
2	60	5	59.035	4.827	98.39	96.54
3	60	5	61.352	4.919	102.25	98.38
4	60	5	58.376	4.961	97.29	99.22
5	60	5	58.564	4.805	97.60	96.10
Mean			59.854	4.869	99.75	97.38
±SD			1.6672	0.0672	2.7787	1.3455
%RSD			2.7855	1.3817	2.7855	1.3817

5.2 METHOD VALIDATION

5.2.1 ACCURACY

Table: 5.10. Results of Accuracy by RP-HPLC Method

Level of % Recovery	Sr. No.	Label claim (µg/tab)		Amount taken (µg)		Amount of Standard Drug Added (µg)		Total Amount Recovered (µg)		%Recovery	
		AMH	LOR	AMH	LOR	AMH	LOR	AMH	LOR	AMH	LOR
80%	1	60	5	120	10	96	8	94.0776	8.0501	97.9975	100.626
	2	60	5	120	10	96	8	95.1941	7.9866	99.1605	99.8329
	3	60	5	120	10	96	8	97.8800	7.9498	101.9583	99.3736
100%	1	60	5	120	10	120	10	120.9247	9.8190	100.7705	98.1904
	2	60	5	120	10	120	10	117.7447	10.0272	98.12058	100.2722
	3	60	5	120	10	120	10	121.0352	9.99498	100.8627	99.9498
120%	1	60	5	120	10	144	12	145.3941	12.0818	100.9681	100.626
	2	60	5	120	10	144	12	141.9258	11.9571	98.5596	99.8329
	3	60	5	120	10	144	12	143.3482	11.9877	99.5473	99.3736

Table: 5.11 Statistical Validation Data for Accuracy

Level of % Recovery	Mean* (% Recovery)		±SD		%RSD	
	AMH	LOR	AMH	LOR	AMH	LOR
80%	99.7054	99.9443	2.0358	0.6337	2.0418	0.6341
100%	99.9179	99.4710	1.5572	1.1207	1.5585	1.1267
120%	99.6917	100.074	1.2107	0.5416	1.2144	0.5412

*Mean of 3 Estimations

5.2.2 PRECESION

Table: 5.12 Results of Intra-day Precision of AMH for RP-HPLC Method

Concentration (µg/ml)	Peak Area Ratio at Following Time (hr)			Mean	±SD	%RSD
	0	2	4			
20	1.7526	1.7839	1.793	1.7765	0.0211	1.1929
40	3.5262	3.476	3.3938	3.4653	0.0668	1.9288
100	4.9485	5.1078	4.9229	4.9930	0.1001	2.0064

Table: 5.13 Results of Intra-day Precision of LOR for RP-HPLC Method

Concentration (µg/ml)	Peak Area Ratio at Following Time (hr)			Mean	±SD	%RSD
	0	2	4			
2	0.3578	0.3497	0.3497	0.3524	0.0046	1.3270
4	0.7155	0.7242	0.7121	0.7172	0.0062	0.8700
10	1.1021	1.0703	1.0484	1.0736	0.0270	2.5150

Table: 5.14 Results of Inter-day Precision of AMH for RP-HPLC Method

Concentration (µg/ml)	Peak Area Ratio at Following day			Mean	±SD	%RSD
	1	2	3			
20	1.6831	1.7547	1.6957	1.7111	0.0382	2.2337
50	4.0984	4.1891	4.1964	4.1613	0.0545	1.3119
200	16.9558	16.8600	16.8345	16.8834	0.0639	0.3788

Table: 5.15 Results of Inter-day Precision of LOR for RP-HPLC Method

Concentration (µg/ml)	Peak Area Ratio at Following day			Mean	±SD	%RSD
	1	2	3			
2	0.3518	0.3494	0.3486	0.3499	0.0016	0.4759
5	0.8837	0.9121	0.8978	0.8978	0.0142	1.5815
20	3.5881	3.5929	3.4954	3.5588	0.0549	1.5442

5.2.3 REPEATABILITY

Table: 5.16 Results of Repeatability (RP-HPLC Method)

Drugs	Conc. (µg/ml)	Peak Area Ratio							±SD	%RSD
		I	II	III	IV	V	VI	Mean		
AMH	30	2.539	2.547	2.543	2.689	2.670	2.522	2.5570	0.0277	1.0866
	50	4.189	4.189	4.201	4.211	4.250	4.192	4.2133	0.0323	0.7669
LOR	3	0.537	0.545	0.542	0.547	0.549	0.539	0.5426	0.0060	1.1107
	5	0.932	0.889	0.892	0.901	0.912	0.923	0.9023	0.0061	0.6771

5.2.4 REPRODUCIBILITY

Table: 5.17 Results of Reproducibility (RP-HPLC Method)

Drugs (conc^{n})	(Peak Area Ratio*±SD)		Result of F-test	Result of t-test$^{\#}$	Inference
	Analyst 1	Analyst 2			
AMH(50)	4.2133±0.0323	4.1994±0.0542	0.3448	0.3861	No significant difference
LOR (5)	0.9023±0.0061	0.8988±0.0102	0.3700	1.9891	No significant difference

*Mean of 3 Estimations # tabulated (standard) value is 9.00 at probability level 0.10

5.2.1 SYSTEM SUITABILITY

Table: 5.18 Results of System Suitability Parameters

Analyte	Retention Time* (min)	Tailing Factor* (T)	Theoretical Plates* (N)	Theoretical Plates* / 250 mm	Resolution* (R)
CAF	2.173	1.895	2451	9803	--
AMH	2.517	1.783	2578	10312	1.942
LOR	13.557	1.704	5028	20112	22.989
Required limits	--	T < 2	N > 2000	--	R >2

*Mean of 6 Estimations

5.2.2 ROBUSTNESS

Table: 5.19 Result of Robustness for Variation in pH

pH	Analyte	Retention Time* (min)	Tailing Factor (T)	Theoretical Plates (N)	Theoretical Plates / 250 mm	Resolution(R)
4.85#	CAF	2.147	1.789	2894	11577	--
	AMH	2.440	1.857	2681	10724	1.664
	LOR	12.557	1.772	4986	19924	22.584
5.00#	CAF	2.173	1.895	2451	9803	--
	AMH	2.517	1.783	2578	10312	1.942
	LOR	13.557	1.704	5028	20112	22.989
5.15#	CAF	2.153	1.950	2749	10996	--
	AMH	2.517	1.917	2732	10927	2.042
	LOR	13.830	1.202	5643	22573	24.420

* % RSD was found to be less than 3 for each drug; #Mean of 3 Estimations

Table: 5.20 Result of Robustness for Variation in Flow Rate (ml/min)

Flow Rate (ml/min)	Analyte	Retention Time* (min)	Tailing Factor (T)	Theoretical Plates (N)	Theoretical Plates / 250 mm	Resolution (R)
1.455#	CAF	2.207	1.762	2378	9513	--
	AMH	2.517	2.042	2430	9721	1.679
	LOR	13.163	1.777	4855	19418	22.224
1.5#	CAF	2.173	1.895	2451	9803	--
	AMH	2.517	1.783	2578	10312	1.942
	LOR	13.557	1.704	5028	20112	22.989
1.545#	CAF	2.083	1.737	2573	10293	--
	AMH	2.373	2.273	5429	9718	1.648
	LOR	12.453	1.798	4794	19177	22.164

* % RSD was found to be less than 3 for each drug; #Mean of 3 Estimations

5.3 FORCED DEGRADATION STUDY

5.3.1 ACID DEGRADATION

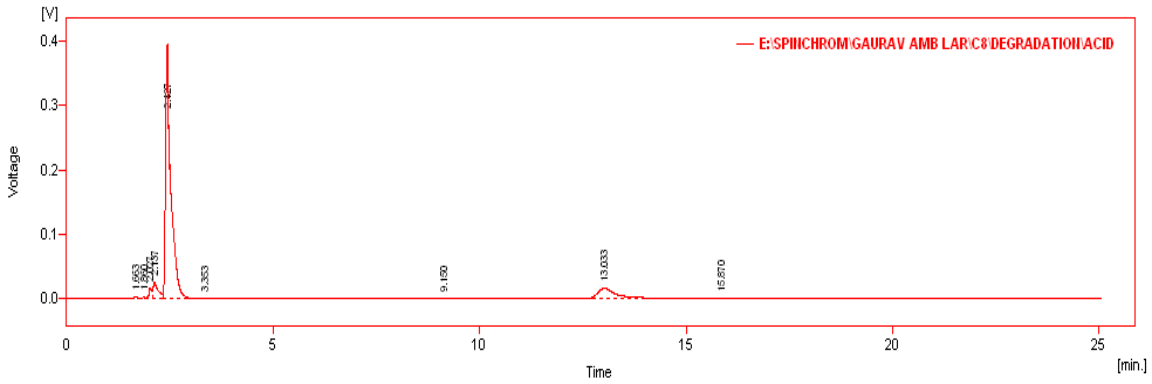


Fig.5.21 Chromatogram of combination of standard drugs in acid degradation (1N HCl, 45min)

Table 5.21 Result of Chromatogram of standard drug in acid degradation

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.663	3.235	0.5
Deg-2	2.027	17.277	1.9
CAF	2.137	24.964	4.5
AMH	2.427	451.460	79.7
LOR	13.033	84.760	12.6

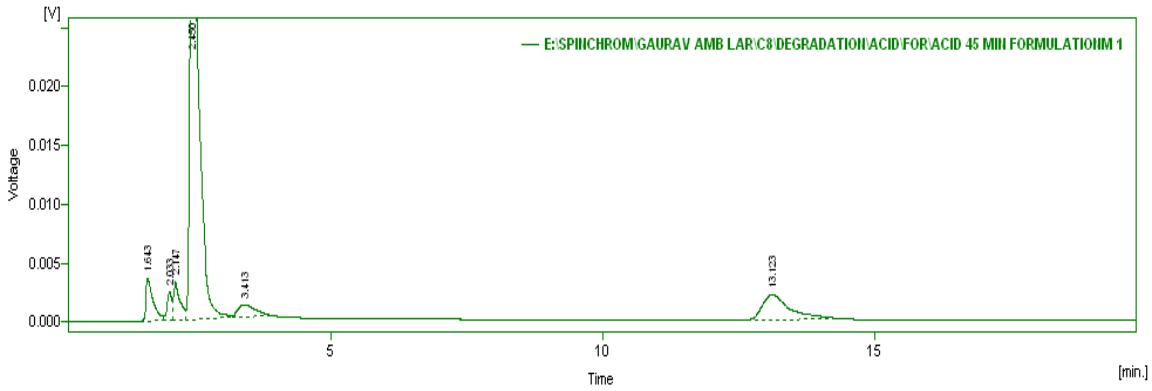


Fig.5.22 Chromatogram of tablet preparation in acid degradation (1N HCl, 45min)

Table 5.22 Result of Chromatogram of tablet preparation in acid degradation (1N HCl, 45min)

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.643	33.019	4.7
Deg-2	2.033	12.585	1.8
CAF	2.147	28.699	4.1
AMH	2.450	527.364	74.9
LOR	13.123	100.517	10.9

5.3.2 BASE DEGRADATION

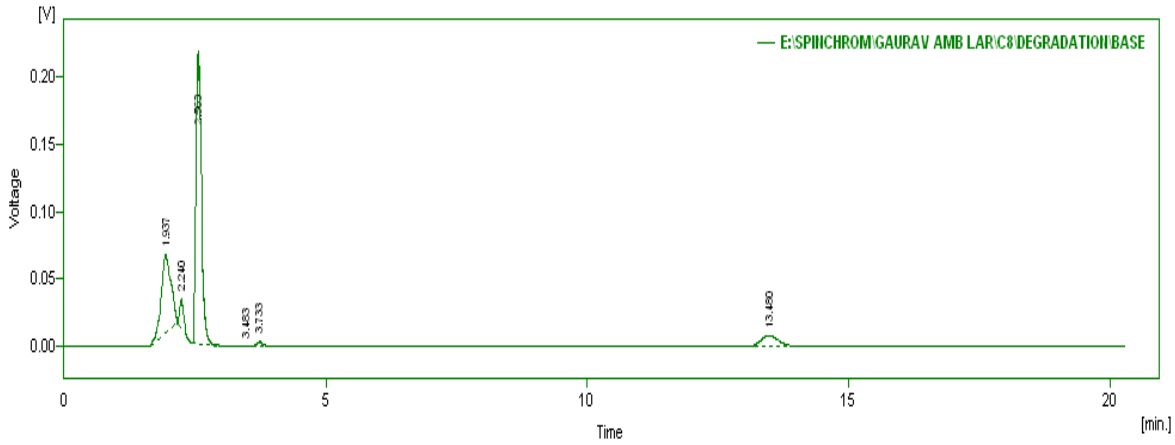


Fig.5.23 Chromatogram of combination of standard drugs in base degradation (1N NaOH, 60min)

Table 5.23 Result of Chromatogram of standard drugs in base degradation

Name	Retention time (min)	Area(mV.sec)	%Area
Deg-1	1.937	721.924	28.1
CAF	2.240	88.821	4.213
AMH	2.563	1491.374	216.273
Deg-2	3.733	30.519	1.2
LOR	13.480	267.031	8.5

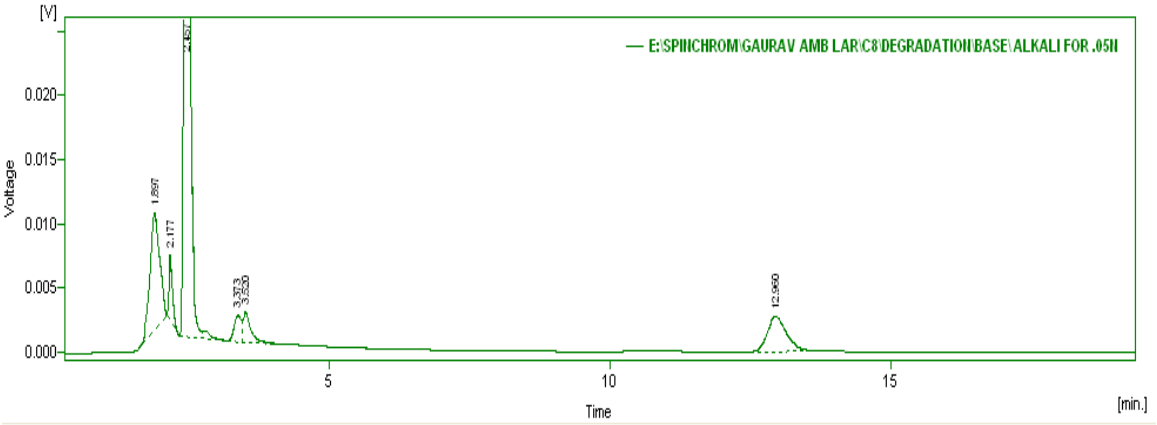


Fig.5.24 Chromatogram of tablet preparation in base degradation (1N NaOH, 60min)

Table 5.24 Result of chromatogram of tablet preparation in base degradation

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.897	103.061	15.3
CAF	2.177	25.800	3.8
AMH	2.457	436.212	64.9
Deg-2	3.373	16.363	2.4
Deg-3	3.520	24.754	3.7
LOR	12.960	75.974	9.8

5.3.3 OXIDATIVE DEGRADATION

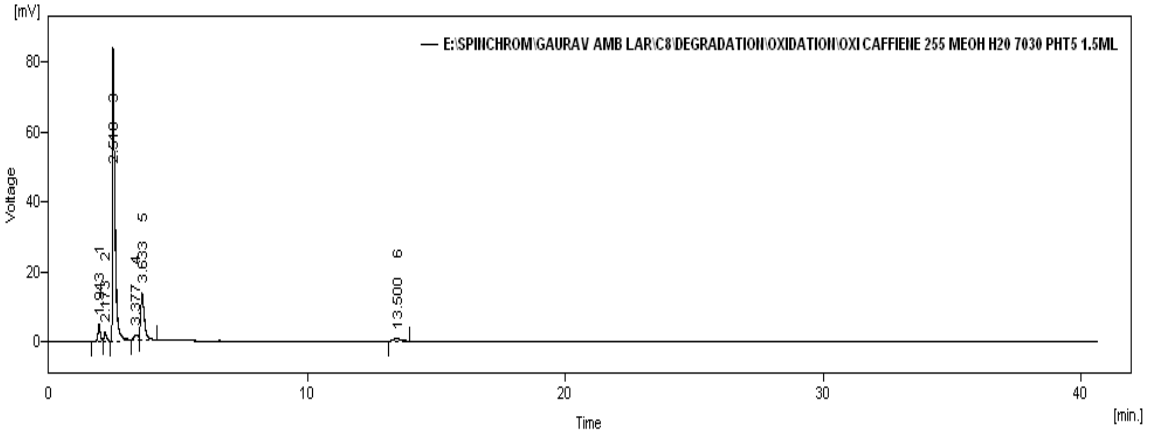


Fig.5.25 Chromatogram of combine of standard drugs in oxidative degradation (3%H_2O_2, 30min)

Table 5.25 Result of Chromatogram of standard drugs in oxidative degradation

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.943	31.022	3.6
CAF	2.173	17.025	2.0
AMH	2.510	360.510	70.2
Deg-2	3.377	18.406	2.2
Deg-3	3.633	99.092	15.4
LOR	13.500	51.770	6.2

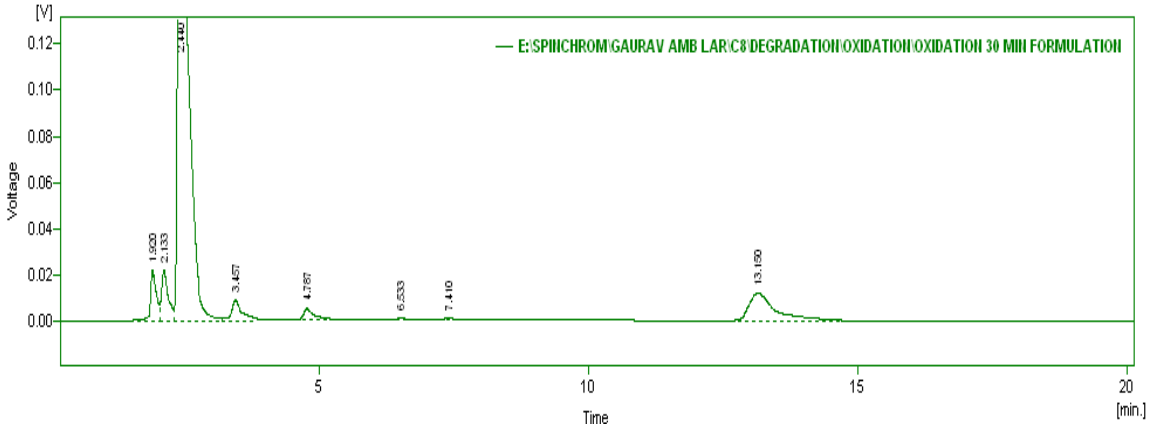


Fig.5.26 Chromatogram of tablet preparation in oxidative degradation (3%H_2O_2, 30min)

Table 5.26 Result of Chromatogram of tablet preparation in oxidative degradation (3%H_2O_2, 30min)

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.920	160.155	3.3
CAF	2.133	180.230	3.7
AMH	2.440	3854.494	79.5
Deg-2	3.457	131.741	2.7
Deg-3	4.787	78.903	1.6
Deg-4	6.533	11.129	0.2
LOR	13.150	569.492	8.6

5.3.4 REDUCTIVE DEGRADATION

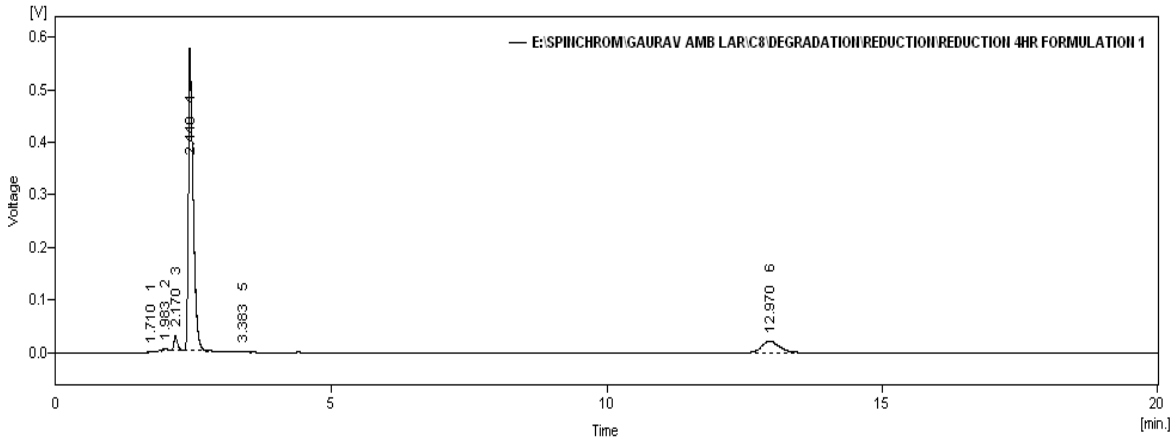


Fig.5.27 Chromatogram of combine of standard drugs in reductive degradation (10%$NaHSO_3$, 4 Hour)

Table 5.27 Result of Chromatogram of standard drugs in reductive degradation

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.903	23.312	7.6
CAF	2.177	8.712	3.5
AMH	2.497	213.720	70.4
Deg-2	3.370	27.179	8.9
LOR	13.233	30.492	9.6

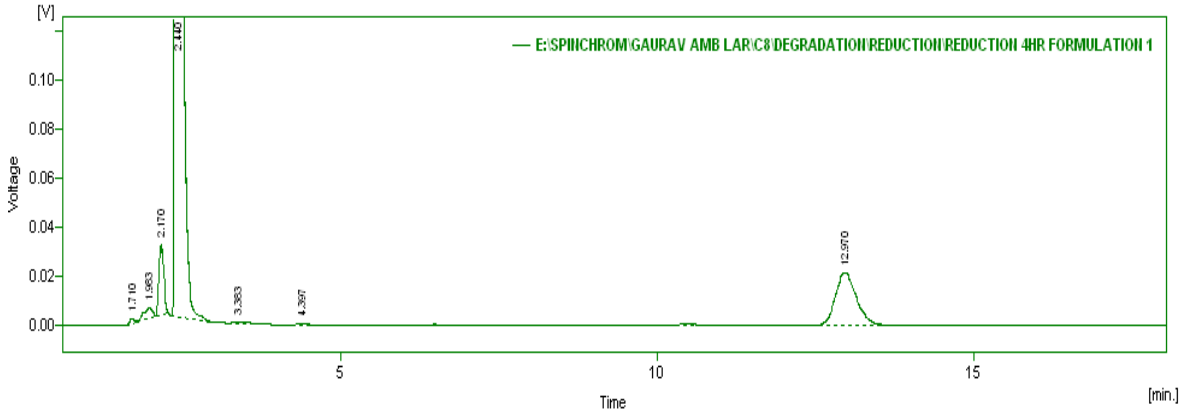


Fig.5.28 Chromatogram of tablet preparation in reductive degradation (10%$NaHSO_3$, 4 Hour)

Table 5.28 Result of Chromatogram of tablet preparation in reductive degradation (10%$NaHSO_3$, 4 Hour)

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.710	8.266	0.2
Deg-2	1.983	39.987	0.9
CAF	2.170	144.329	3.2
AMH	2.440	3682.386	83.8
Deg-3	3.383	14.040	0.3
LOR	12.970	503.66	11.2

5.3.5 NEUTRAL DEGRADATION

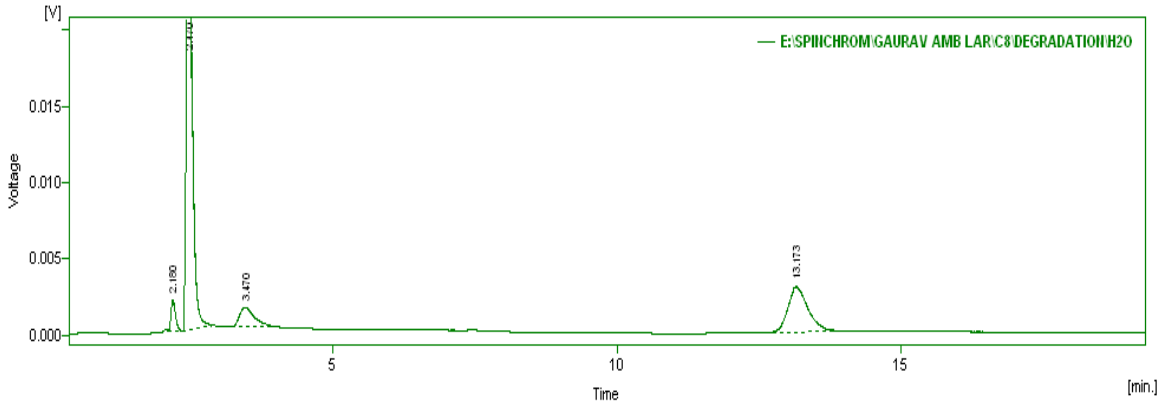


Fig.5.29 Chromatogram of combination of Standard drugs in neutral degradation (Water, 4 Hour)

Table 5.29 Result of Chromatogram of Standard drugs in neutral degradation

Name	Retention time (min)	Area (mV.sec)	%Area
CAF	2.180	10.537	3.3
AMH	2.470	211.110	66.5
Deg-1	3.470	23.524	7.4
LOR	13.173	47.788	22.6

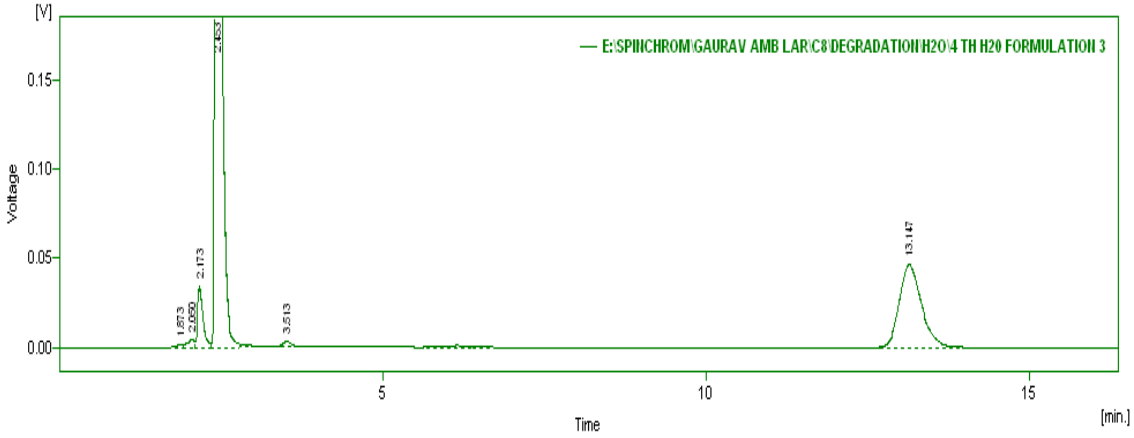


Fig.5.30 Chromatogram of tablet preparation in neutral degradation (Water, 4 Hour)

Table 5.30 Result of chromatogram of tablet preparation in neutral degradation (Water, 4 Hour)

Name	Retention time (min)	Area (mV.sec)	%Area
Deg-1	1.873	11.324	0.3
Deg-2	2.050	33.452	0.7
CAF	2.173	201.990	4.5
AMH	2.453	3658.027	68.5
Deg-3	3.513	33.677	0.8
LOR	13.147	901.26	24.8

Table 5.31 Results of forced degradation study of mixture of standard drugs by proposed RP-HPLC method

Stress condition/Strength duration	Drugs	% Assay of drugs after degradation	% Degradation
Acidic/ 1N HCl/ 45 min	AMH	70.9193	29.0807
	LOR	75.6189	24.3811
Basic/ 0.5N NaOH/ 60 min	AMH	65.8462	34.1538
	LOR	66.9575	33.0425
Oxidative/ 3% H_2O_2/ 30 min	AMH	83.0405	16.9595
	LOR	67.7243	32.2757
Reductive/ 10% $NaHSO_3$/ 4 hr	AMH	96.2026	3.7974
	LOR	77.9510	22.0490
Neural/ water/ 4 hr	AMH	78.5690	21.4310
	LOR	100	0

Table 5.32 Results of forced degradation study of tablet formulation by proposed RP-HPLC method

Stress condition/Strength duration	Drugs	% Assay of drugs after degradation	% Degradation
Acidic/ 1N HCl/ 45 min	AMH	72.0615	27.9385
	LOR	78.0057	21.9943
Basic/ 0.5N NaOH/ 60 min	AMH	66.3036	33.6964
	LOR	65.5841	34.4159
Oxidative/ 3% H_2O_2/ 30 min	AMH	83.8687	16.1313
	LOR	70.3743	29.6257
Reductive/ 10% $NaHSO_3$/ 4 hr	AMH	100	0
	LOR	77.7208	22.2792
Neural/ water/ 4 hr	AMH	71.0193	28.9807
	LOR	100	0

6. DISCUSSION

6. DISCUSSION

6.1 REVERSE PHASE HIGH PERFORMANCE LIQUID CROMATOGRAPHY

6.2 METHOD VALIDATION

6.3 FORCED DEGRADATION STUDY

6.1 REVERSE PHASE HIGH PERFORMANCE LIQUID

Optimization of Chromatographic Conditions

Mixed solution of AMH (50 µg/ml), LOR (10 µg/ml) and CAF (10 µg/ml) was prepared and injected into the HPLC system. The solution was analyzed using different mobile phases like Acetonitrile: Ammonium Acetate (50:50 and 60:40% v/v at pH 2.0, 3.0 and 7.0), Acetonitrile: water (60:40% v/v at pH 2.0, 3.0, 6.0, 6.4 and 7.0) using C_{18} column at different flow rate such as 1.0 and 1.2 ml/min. The mixture of methanol and water in proportion of 50:50% v/v (pH 2) was tried for separation of mixture of drugs and internal standard on C_{18} column at different flow rates. The chromatogram of mixed solution of drug and internal standard was also studied on C_8 column using methanol: 0.1% OPA(70:30 % v/v, pH 2, 5, 8) at different flow rate 1, 1.2, 1.5 ml/min. Retention time, peak area, tailing factor, theoretical plates and resolution were observed for each peak of the chromatogram. It was found that the Mobile Phase consisting of methanol: 0.1% OPA (70:30%v/v,) adjusting the pH 5.0 with 0.1% triethylamine solution showed good results at flow rate of 1.5ml/min on C_8 column. So, these conditions were selected for the analysis of the drugs.

Selection of Analytical Wavelength

The standard solutions of AMH (50 µg/ml), LOR (10 µg/ml) and CAF (10 µg/ml) in MP were scanned in the UV region of 400 to 190 nm using mobile phase as blank and the overlain spectra were recorded. It was observed that all the three drugs observed prominently at 255 nm, hence this wavelength was used for the measurement of absorption.

Selection of pH

The pKa value of AMH and LOR is 8.2 and 4.9 respectively. A number of pH was tried but more rugged mobile phase pH was to be appeared to 5.0. At this pH, peak tailing was minimum and resolution between degrade products and drugs were found to be optimum. So, pH of mobile phase was adjusted to 5.0 with triethylamine solution.

Calibration

AMH and LOR were found to be linear in the concentration range of 10-200 µg/ml and 1-20 µg/ml, respectively.

Assay

Amount of drugs present in the marketed formulation (Lorfast-AM) were calculated using equations mentioned in the section no. 4.3.5. Amount of AMH and LOR were found in the range from 97.29 – 103.23% and 96.10 – 99.20%, respectively.

Method Validation

This method was validated in accordance to ICH guidelines. Recovery studies were carried out by standard addition method by adding the known amount of AMH and LOR (reference standard) to the preanalyzed sample at three different concentration levels i.e. 80%, 100%, and 120% of assay concentration and percentage recoveries were calculated. Percentage of recoveries of AMH and LOR were found in the range from 97.99 – 101.95% and 99.37 – 100.62%, respectively. Precision of the method was determined by % RSD found among intra-day precision, inter-day precision, repeatability. It was found to be less than

3 %. Reproducibility was determined by preparing and measuring the standard solutions of AMH (50 µg/ml) and LOR (5 µg/ml) by Analyst 1 and Analyst 2, separately. The values obtained were evaluated using F-test and t-test to verify their reproducibility. Calculated value for t-test was found to be less than the tabulated (standard) value it can calculated that no significant difference was observed in the result of analysis. Detection limit and quantitation limit were determined from the standard deviation of y – intercepts of six calibration curves and average slope of six calibration curves. LOD and LOQ of AMH were found to be 3.4866 and 10.564 µg/ml, respectively. LOD and LOQ of LOR were found to be 0.5806 and 1.759 µg/ml, respectively.

For robustness study, the effect of change in the pH of mobile phase and flow rate on the retention time, tailing factor, theoretical plates and resolution were studied. Combined standard solutions of AMH (50µg/ml), LOR (5 µg/ml) with CAF (10 µg/ml) were prepared and analyzed at different pH (4.85, 5.00, 5.15) of the mobile phase and at different flow rate (1.455, 1.500, 1.545 ml/min). Percentage RSD of retention time of each peak was found to be less than 1 %.

Forced Degradation Study

AMH and LOR were exposed to different stress conditions such as acidic, basic, oxidative, reductive and neutral for different strengths and refluxed on heating mantle for different time periods. The developed RP-HPLC method was used for quantitation of drug in presence of degraded products. The amount of degradation of both drugs were found to be in range of 10-30% at the end of 45 min in 1N HCl, 1 hr in 0.5N NaOH, 30 min in 3% H_2O_2, 4 hr in 10% w/v $NaHSO_3$, 4 hr in

water while more than 30% degradation was observed in 0.5N NaOH at end of 1hr. So these conditions were selected for formulation degradation. There was no degradation found for AMH and LOR in reductive and neutral condition respectively.

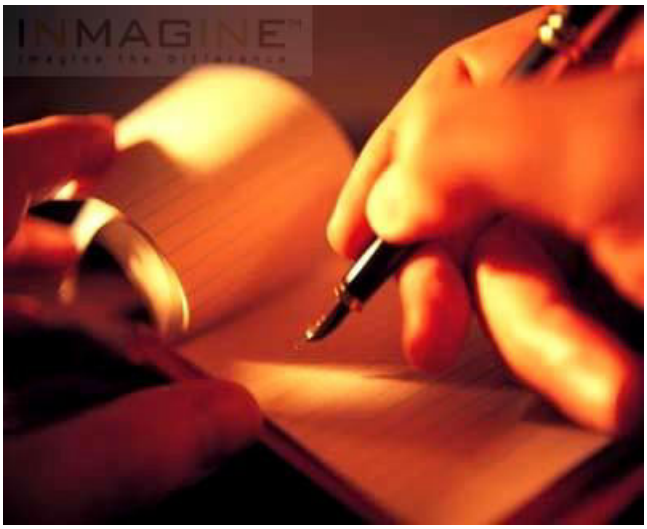
Based on results, obtained from the analysis of forced degradation samples using proposed method, it can be concluded that developed RP-HPLC method with an internal standard will successfully applicable for simultaneous estimation of AMH and LOR in presence of their degraded product in pharmaceutical formulation.

7. CONCLUSION

7. Conclusion:

The developed stability indicating RP-HPLC method was found to be simple, accurate, sensitive, precise, specific and rapid. This method can be applied for routine quantitative analysis of Ambroxol hydrochloride and Loratadine in bulk and pharmaceutical formulations like tablets. This method was also capable to separate the degradation product of both drugs hence it can be used to check quality of product after different storage condition and in stress degradation study.

8. SUMMARY



8. SUMMARY

An attempt has been made to develop and validate the Stability indicating HPLC Method for the simultaneous estimation of Ambroxol hydrochloride and Loratadine in pharmaceutical formulation.

RP-HPLC Method was performed according to the chromatographic conditions mentioned in the following Tables.

Table: 8.1. HPLC System

Liquid Chromatograph	Shimadzu LC–20AT
UV–Visible Detector	Shimadzu SPD–20A
Analytical Column	Phenomenex C_8 (250 mm × 4.6 mm, 5 μm)
Data Processor	Spinchrome CFR Software
Injector	Rheodyne – 7725i (Fixed Capacity Loop of 20 µl)
Syringe	Hamilton, 25 µl

Table: 8.2. Chromatographic Conditions

Mobile Phase	Methanol: 0.1%OPA (60:40 % v/v)
pH of Mobile phase	5.0 adjusted with 0.1% triethylamine solution
Internal Standard (IS)	Caffeine (10 µg/ml)
Flow Rate	1.5 ml/min
Detection Wavelength	255 nm
AUFS	0.1000
Pressure	18.9 Mpa

The developed RP-HPLC method for estimation of AHM and LOR using CAF as an internal standard was validated in accordance with ICH guidelines. The validation results are summarized in table 8.3 and 8.4.

Table: 8.3. Summary of Linear Regression Analysis of Calibration Curves for AMH and LOR for RP-HPLC Method

Parameters	AMH	LOR
Linearity Range (µg/ml)	10-200	1-20
Slope	0.085	0.1796
Intercept	0.0898	0.0316
Correlation Coefficient (r^2)	0.9996	0.9992
LOD (µg/ml)	2.2866	0.4530
LOQ (µg/ml)	6.9299	0.8728

Table: 8.4. Summary of Assay Results and Validation Parameters for RP-HPLC Method

Parameters	AMH	LOR
Analysis of Tablets (% Assay)	97.29 – 103.23%	96.10 – 99.20%
% Recovery	99.70-99.91%	99.47-100.04%
Intra Day Precision (%RSD)	1.1929-2.0064	0.8700-2.5150
Inter Day Precision (%RSD)	0.3788-2.2337	0.4759-1.5815
Repeatability (±RSD)	0.7669-1.0866	0.6771-1.1107
Reproducibility# (t-test)	0.3861	1.9891
Robustness* (%RSD)	< 3 %	< 3 %

tabulated (standard) value is 9.00 at probability level 0.10

Forced degradation study was performed using different stress conditions such as acidic, basic, oxidative, reductive and neutral for API and formulation.

The results of forced degradation study of API and formulation were mentioned in following table.

Table: 8.5. Summary of Forced Degradation Study Results of API and Formulation by RP-HPLC Method

Stress condition/Strength duration	Drugs	API		Marketed formulation	
		% Assay of drugs after degradation	% Degradation	% Assay of drugs after degradation	% Degradation
Acidic/ 1N HCl/ 45 min	AMH	70.9193	29.0807	72.0615	27.9385
	LOR	75.6189	24.3811	78.0057	21.9943
Basic/ 0.5N NaOH/ 60 min	AMH	65.8462	34.1538	66.3036	33.6964
	LOR	66.9575	33.0425	65.5841	34.4159
Oxidative/ 3% H_2O_2/ 30 min	AMH	83.0405	16.9595	83.8687	16.1313
	LOR	67.7243	32.2757	70.3743	29.6257
Reductive/ 10% $NaHSO_3$/ 4 hr	AMH	96.2026	3.7974	100	0
	LOR	77.9510	22.0490	77.7208	22.2792
Neural/ water/ 4 hr	AMH	78.5690	21.4310	71.0193	28.9807
	LOR	100	0	100	0

From present study, it can be concluded that the developed RP-HPLC method with the used of internal standard will be successfully applicable for simultaneous estimation of Ambroxol hydrochloride and Loratadine in presence of their degraded product in pharmaceutical industry.

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